AD_____

Award Number: W81XWH-08-1-0372

TITLE: Age-Related DNA Methylation Changes and Neoplastic Transformation of the Human Prostate

PRINCIPAL INVESTIGATOR: Bernard Kwabi-Addo, Ph.D.

CONTRACTING ORGANIZATION: Howard University
Washington D.C. 20060

REPORT DATE: July 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 Jul 2011	Final	15 JUN 2008 - 14 JUN 2011

4. TITLE AND SUBTITLE

Age-Related DNA Methylation Changes and Neoplastic Transformation of the Human Prostate

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0372

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Bernard Kwabi-Addo, Ph.D.

E-Mail: bkwabi-addo@howard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Howard University
Washington D.C. 20060

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

There is abundant evidence to suggest that epigenetic DNA methylation changes may appear early during prostate cancer (PCa) development than genetic changes, and are more common and consistent. The purpose of this study is to investigate whether aberrant DNA methylation occurs as a function of age and if it accompanies neoplastic transformation of the human prostate. RESULTS: Using methylated CpG island amplification coupled with promoter microarray and also genome-wide DNA methylation analysis, I have identified several novel genes that are differentially methylated in normal and PCa tissues. In addition, I have also identified novel methylated patterns among pre-invasive and cancerous lesions of the prostate. Quantitative methylation analysis revealed significant differences ($p < 0.05$) in methylation level for several genes in African-American (AA) samples versus Caucasian (Cau) samples. Furthermore, regression analysis revealed significantly higher methylation for NKX2-5 ($p = 0.008$) and TIMP3 ($p = 0.039$) genes in normal prostate tissue samples from AA versus Cau, and a statistically significant association of methylation with age for NKX2-5 ($p = 0.03$) in AA versus Cau. In addition, functional studies demonstrate that NKX2-5 may have tumor suppressor function in PCa cells. CONCLUSION: I have identified several novel methylated genes as potential (ethnic sensitive) biomarkers for early disease detection and for distinguishing indolent from aggressive PCa.

15. SUBJECT TERMS
Dna Methylation; Genome Wide Methylation Analysis, Pyrosequencing, Rt-Pcr, Western Blot Analysis, African-American, Caucasian Men, Prostate Cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	124	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

Prostate cancer (PCa) is a common malignancy and a leading cause of cancer death among men in the United States. Racial differences in PCa incidence and mortality are well documented. The incidence and mortality for PCa is about twofold higher in African American (AA) in comparison with Caucasian (Cau) men, with AA men experiencing among the highest rates worldwide[1]. African American men have a 60% increased risk of developing prostate cancer, twice the risk of developing distant disease and twice the mortality relative to their Caucasian counterpart [2]. The disparity in PCa is believed to be a complex combination of socioeconomic factors, environment and genetics [3]. Genetic studies of prostate cancer cells shows a variety of genetic defects, including gene mutations, deletions, translocations, and amplifications, that endow the cells with new capabilities for dysregulated proliferation, inappropriate survival, tissue invasion and destruction, immune system evasion, and metastasis [4]. More recently, it has become apparent that prostate cancer cells also carry epigenetic defects, including changes in cytosine methylation patterns and in chromatin structure and organization, which are equivalent to genetic changes in effecting and maintaining neoplastic and malignant phenotypes [5]. For human prostate cancer, abundant evidence has accumulated to suggest that somatic epigenetic alterations may appear earlier during cancer development than genetic changes, as well as more commonly and consistently. Furthermore, epigenetic changes tend to arise in association with age [6] and/or in response to chronic or recurrent inflammation leading to cell and tissue damage [7].

Epigenetic inactivation of genes in cancer cells is largely based on transcriptional silencing by aberrant CpG methylation of CpG-rich promoter regions [8, 9]. Aberrant

promoter methylation of GSTP1, encoding the π-class glutathione *S*-transferase (GST), an enzyme capable of detoxifying electrophilic and oxidant carcinogens remains the most common somatic genome abnormality of any kind (>90% of cases) reported thus far for prostate cancer, appearing earlier and more frequently than other gene defects that arise during prostate cancer development [10]. Since the recognition that the *GSTP1* CpG was frequently hypermethylated in prostate cancer, more than 40 genes have been reported to be targets of DNA hypermethylation-associated epigenetic gene silencing in prostate cancer cells [11]. Despite the increasing number of aberrantly methylated genes in prostate cancer, only a few genes show promise as prostate cancer biomarkers for early diagnosis and disease risk assessment [12].

Underlying this aberrant DNA methylation is the accumulating body of data hinting that normal prostate cells may be subjected to a relentless barrage of genome-damaging stresses due to both exogenous and endogenous carcinogens, with damage accumulating over time and age. Thus aberrant epigenetic DNA methylation changes may represent the integration of environmental or lifestyle exposures and genetic predisposition to prostate cancer. Such events may differ between individuals belonging the same ethnic group or individuals belonging to different ethnic groups. Thus the elucidation of methylation changes in prostate tissues from different ethnic groups would contribute to our understanding of the molecular mechanisms underlying prostate cancer disparity and potentially lead to the identification of "ethnic sensitive" biomarkers for early disease detection. We know that there are different thresholds for African American versus Caucasian men for PSA screening. Therefore other markers such as DNA methylated genes that can clarify such ethnic sensitive screening strategies would also be helpful.

Furthermore, differential methylation changes could also lead to identification of potential novel therapeutic targets for prostate cancer treatment.

In this project, I sought to investigate if DNA methylation changes occur in human prostate tissues as a function of age and whether DNA methylation may potential contribute to neoplastic transformation of the human prostate tissue samples.

Because aberrant epigenetic DNA methylation changes represents the integration of environmental or lifestyle exposures and genetic predisposition to prostate cancer, differences in DNA methylation patterns in prostate tissue specimens from African American men in comparison to Caucasian men may help in understanding the contribution of DNA methylation changes in creating a more aggressive disease milieu in African American men in comparison with their Caucasian counterparts and to identify candidate DNA methylation genes that could serve as useful biomarker ("ethnic sensitive") for the detection of PCa.

BODY

As outlined in my Statement of work, I sort to accomplish 3 main tasks during the 3 years of funding. I have accomplished all these tasks within the proposed research period. Details of the accomplished proposed tasks as outlined in my statement of work are given below as well as in the attached publications.

Task 1: Acquisition and modification of prostate tissues from Caucasian and African-American Men (months 1-6):

The table 1 below shows the prostate tissue samples obtained from African American and Caucasian men as either organ donors, cystoprostatectomy patients for bladder cancer or radical prostatectomy samples. High molecular weight DNA and RNA were extracted

from prostate tissues. Genomic DNA samples were modified using sodium bisulfite treatment and used for quantitative DNA methylation analysis whereas RNA was converted to cDNA and used for quantitative RT-PCR analysis.

A.

	AA	**Cau**
T2	17 (44)	28 (43)
T3a	18 (46)	31 (58)
T3b	4 (10)	6 (9)
N1	0 (0)	2 (3)
Gleason	6.4	6.8

B.

	African-American (AA)				Caucasian (Cau)			
#	Core	PCa GS	PSA (ng/ml)	Age (years)	Core	PCa GS	PSA (ng/ml)	Age (years)
1	RM	NEG (GS=0)	0.5	54	LM	NEG (GS=0)	1.5	65
2	LM	NEG (GS=0)	0.2	63	LA	NEG (GS=0)		58
3	LA	NEG (GS=0)	6.3	60	LM	NEG (GS=0)	4.1	65
4	LM	NEG (GS=0)	4.9	65	LM	NEG (GS=0)	6.2	59
5	LA	NEG (GS=0)	3.6	68	LM	NEG (GS=0)	3.8	60
6	LM	NEG (GS=0)	1.5	71	LM	NEG (GS=0)	4.1	59
7	LM	NEG (GS=0)	5.7	61	LM	NEG (GS=0)	5.1	76
8	LM	NEG (GS=0)	4.8	47	LM	NEG (GS=0)	4.9	64
9	LM	NEG (GS=0)	0.5	67	LM	NEG (GS=0)	8.7	85
10	LM	NEG (GS=0)	6.2	65	LM	NEG (GS=0)	9.0	56
11	LM	NEG (GS=0)	1.4	65	LM	NEG (GS=0)	4.9	72
12	LM	NEG (GS=0)	4.9	70	LM	NEG (GS=0)	2.1	59
13	LM	NEG (GS=0)	4.7	59	LM	NEG (GS=0)	1.7	55
14	LM	NEG (GS=0)	7.7	54				
15	LM	NEG (GS=0)	9.3	61				
16	LM	NEG (GS=0)	14.1	55				
17	LM	NEG (GS=0)	5.0	76				
18	LM	NEG (GS=00	5.0	68				

C.

	Caucasian	*African-American*	*Age-Range (years)*
HGPIN	6	2	45-70
PZCancer	4	3	61- 67

D.

40 pairs of matched normal and prostate cancer tissue samples from Caucasian men who had undergone radical prostatectomy.

Table 1: Clinical patient samples characterized by race/age/pathological features. **A** Matched pairs of normal and prostate cancer (PCa) tissue samples obtained from both African American (AA) and Caucasian (Cau) male patients with PCa at the time of radical prostatectomy. The Staging (T) and Gleason score are shown. Number of cases with percentage ( ). N1 is positive pelvic lymph nodes (age range 52-75 years). **B** Needle biopsies normal prostate tissue samples from AA and Cau male patients (age range 47-85 years) with elevated serum PSA level in the presence of an abnormal digital rectal exam. The mean age was 62.7 $\pm$ 7.2 and 62.4 $\pm$ 8.8 (p= 0.89) for AA and Cau groups, respectively. Fifteen of 18 and 12 of 13 biopsies (p= 0.53) were from the left mid-zone (LM) of the prostate of AA and Cau samples respectively. The remaining samples were either from the left apex (LA) or right mid-zone (RM). Gleason score (GS) indicate noncancerous. **C.** Radical prostatectomy samples with high grade prostatic intraepithelial neoplasia (HGPIN) and prostate carcinoma (PZCancer).

Task 2: Screening and validation of 200 differentially methylated genes (months 3-24):

Several genes have been shown to be hypermethylated in prostate cancer [13]. However, hypermethylation of genes in normal prostate tissues may be an early event predisposing cells to neoplastic transformation. I have recently shown that some genes which are hypermethylated in prostate cancer tissue may undergo methylation in normal prostate tissues in an age-dependent manner. To my knowledge, this was the first study to directly examine the relationship between methylation and age in human prostate tissues. This study clearly demonstrated that methylation starts in normal prostate tissues as a function of age and markedly increases in cancer [6].

A global profile of genes that are methylated in normal prostate tissues as a function of age would serve to identify candidate genes that are hypermethylated as an early event in the transition from normal prostate cells to prostate cancer and ultimately provide insight into understanding the molecular mechanisms underlying DNA methylation, aging and neoplastic transformation. In this aim, I proposed to carry out a comprehensive analysis of novel methylated genes that I have identified using a combination of methylated CpG

island amplification coupled with CpG promoter microarray to ascertain if methylation status can provide reliable information for the detection of prostate cancer.

Detection of Methylated CpG Island Using MCA-CpG promoter microarray

From my preliminary studies, I have used methylated CpG island amplification (MCA) technique [14] coupled with CpG promoter microarray to identify several novel methylated genes in the human prostate cancer line LNCaP. I identified more than 300 differentially hypermethylated loci of which approximately 50 were unique promoter associated CpG islands. Similarly, I identified about 374 differentially hypomethylated loci in the LNCaP cells. Interestingly, 349 of these hypomethylated probes mapped to repetitive elements and only 25 loci were unique promoter associated CpG islands as revealed by BLAST (www.ncbi.nlm.nih.gov) and BLAT (genome.ucsc.edu) searches. Several of these genes have previously been reported [13]. Novel methylated genes of particular interest in the current study included PAX9, RPRM, CDH11, SPARC, FOXN4, TIMP3, and TCF3. These genes were chosen for initial studies based on either their chromosomal localization, their regulatory function or whether they may be important in prostate cancer etiology as shown in Table 2.

No.	Gene name	Bidirectional	Locus	Location	Previously reported
1.	PAX9	No	14q13.3	36202075 - 36202382	Yes
2.	RPRM	No	2q23.3	154042696-154044004	Yes
3.	CDH11	No	16q22.1	63711958-63715365	No
4.	SPARC	No	5q31.3	151046061-151047060	Yes
5.	FOXN4	No	12q24.11	108231010-108232761	Yes
6.	TIMP3	No	22q12.3	31527381 - 31528267	Yes
7.	TCF3	No	19p13.3	1596918-1598213	No

Table 2 shows a list of genes identified as differentially methylated in prostate cancer cell line; LNCaP.

In order to validate the MCA-microarray results, I investigated the methylation status of 10 genes differentially methylated (8 predicted hypermethylated and 2 predicted unmethylated) in a panel of 21 cell lines. To accomplish this, high molecular weight DNA was extracted from the cell lines and modified using sodium bisulfite treatment. Bisulfite PCR primers were designed based on bisulfite/converted sequence from the CpG ensuring that the bisulfite-PCR primers avoid CpG sites and that they are designed as close to the transcription start site as possible. The bisulfite primers were then used in a PCR reaction with the bisulfite treated genomic DNA from the cell lines. A two-step nested PCR reaction was carried out using 2 sets of different PCR primers. This helps improve the specificity and purity of the PCR products used in the pyrosequencing reaction. One of the primers (reverse primer) in the 2^{nd} step PCR reaction was biotinylated in order to create a single-stranded DNA template for the pyrosequencing reaction. The PCR products were immobilized on streptavidin-sepharose beads (Amersham), washed, denatured, and the biotinylated strands released into an annealing buffer containing the sequencing primer. Pyrosequencing was performed using the PSQ HS96 Gold SNP Reagents on a PSQ 96 HS machine (Biotage).

I have carried out the methylation analysis in a 21-cell line panel (Fig 1). All of the genes investigated showed methylation in at least a set of cell lines from one cancer site. Eight of the 10 analyzed genes showed hypermethylation in at least one of the three prostate cancer cell lines. The PAX9 and CYP27B1 genes were unmethylated in prostate cancer cell lines. Individual cell lines showed a range of methylation frequency. The RPRM, SPARC, NKX2-5, RASSF1A genes showed hypermethylation in the prostate cancer cell

lines (I have previously characterized the methylation status of NKX2-5 and RASSF1A in prostate cancer cells and tissues; [15]). The TIMP3, RPRM and RASSF1A also showed hypermethylation in the immortalized prostatic primary epithelial cells (pNT1A cells). It is likely that the methylation observed in pNT1A cells could be derived from repeated passages and selection during cell culture. The CDH11, FOXN4 and CYP27B1 genes did not display hypermethylation in the prostate cell lines. However, individual prostate cancer cell lines showed varied methylation frequency as was observed in the cancer cell lines from different tissue sites.

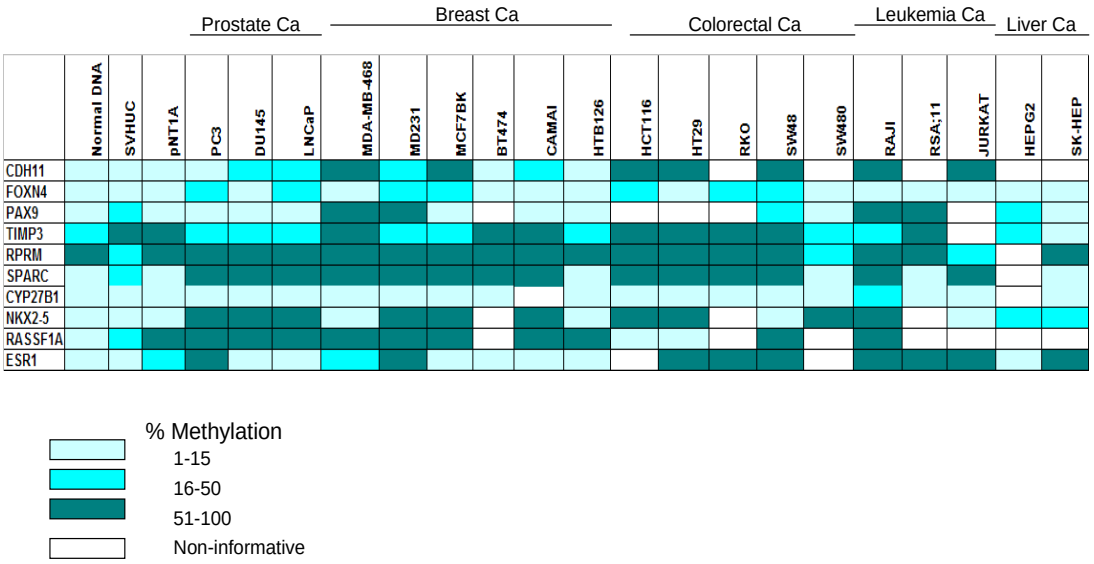


Fig 1. The methylation status of 10 genes was investigated in a 21-cell line panel by pyrosequencing. Cell lines include a tumorigenic urothelial epithelium SVHUC cell line and the primary prostatic epithelial cell line, pNT1a, both immortalized by SV40 transfection. The scale refers to the degree of methylation as measured by pyrosequencing.

DNA methylation analysis in prostate tissues

Having established differential methylation of these genes in the panel of 21-cell lines, I next wanted to compare the level of methylation in normal and prostate cancer tissues. To accomplish this, I obtained matched pairs of benign and prostate cancer tissue samples

from patients who have undergone radical prostatectomy (25 samples). High molecular weight genomic DNA was extracted from the tissue samples. Genomic DNA samples were modified using sodium bisulfite treatment and modified DNA used in pyrosequencing analysis as described above. Results presented in Fig 2 demonstrate that compared with the methylation data from normal prostate tissues, there is significantly higher methylation in prostate cancer tissues for FOXN4, TIMP3, RPRM, SPARC and CYP27B1. On the other hand, CHD11 and PAX9 genes do not show a significant difference in DNA methylation between the normal and matched prostate cancer tissues.

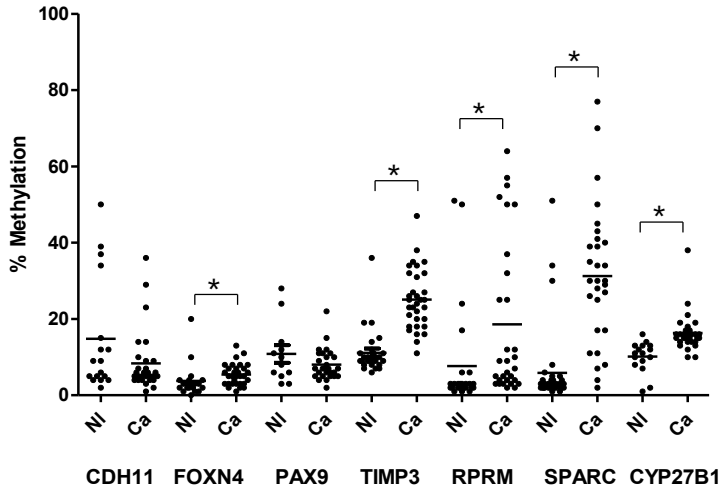


Fig 2. The % methylation level of novel genes in matched normal (Nl) versus prostate cancer (Ca) tissue samples from individual patients that had undergone radical prostatectomy. * shows statistically significant data as determined by Mann Whitney t-test, with significance set at the level of $p < 0.05$.

Methylation and gene expression

To investigate if methylation leads to gene silencing, I performed expression analysis using total RNA extracted from matched normal and prostate cancer tissue samples by quantitative RT-PCR for 6 genes, CDH11, PAX9, TIMP3, SPARC, FOXN4 and RPRM based on their potential function in prostate carcinogenesis as well as their frequency of methylation in cell lines and prostate tissues (Fig 3). I observed a good correlation

between methylation frequency and expression to indicate that methylation leads to gene silencing.

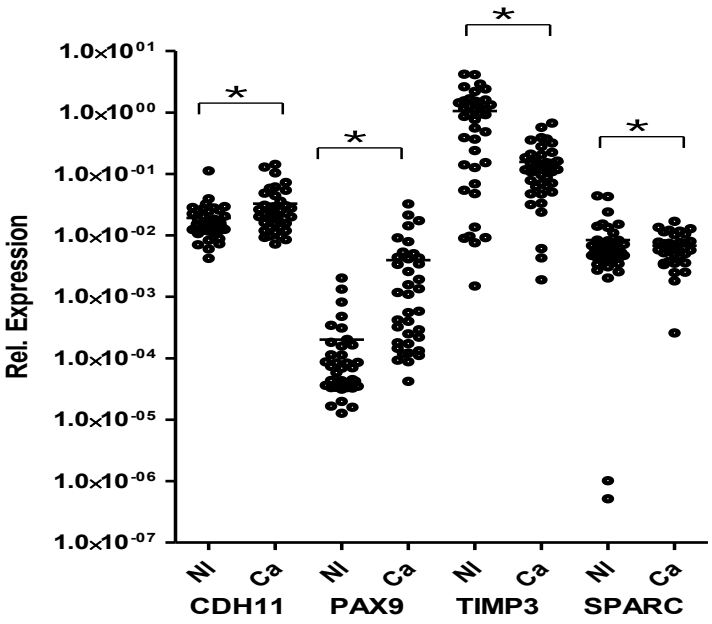


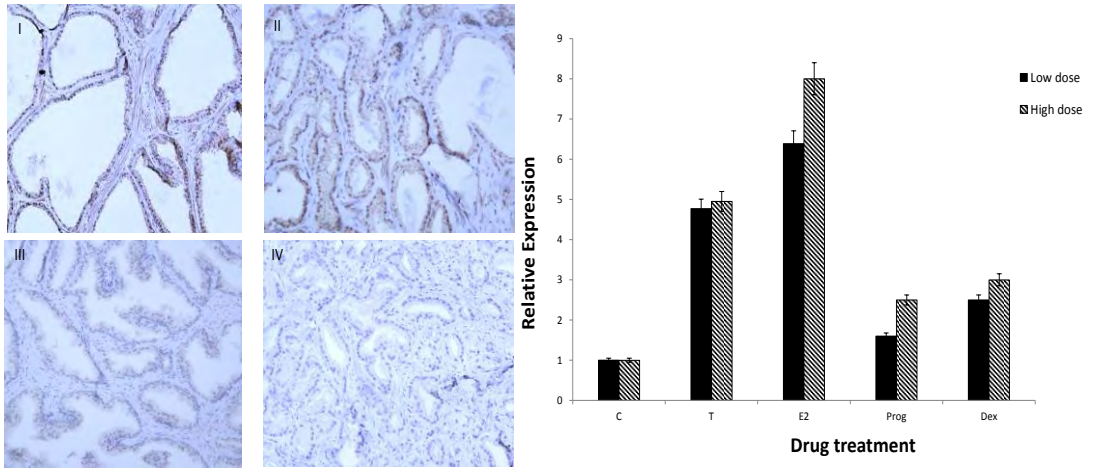
Fig 3. Relative expression of genes in matched normal (Nl) versus prostate cancer (Ca) tissue samples as determined by RT-PCR. * indicates statistically significant data.

Task 3 and 4: The expression profile of differentially methylated genes and evaluation of biological effect in cell culture (Months 25-30):

I have identified NKX2-5, a member of the homeodomain family of transcription factors as a novel DNA methylated gene that shows increase in DNA methylation level as a function of age in normal prostate tissues and also higher DNA methylation prevalence in prostate tissues from AA in comparison to Cau men. Furthermore, NKX2-5 demonstrates a significantly higher DNA methylation level in prostate cancer tissues in comparison with matched benign prostate tissue samples suggesting that DNA methylation of NKX2-5 can be exploited as a potential biomarker for detecting prostate cancer [15].

Expression of NKX2-5 in Normal and Neoplastic Human Prostate Tissue

To gain insight into the cellular localization of NKX2-5 in human prostate tissues, I assessed NKX2-5 expression in 25 pairs of matched benign and prostate cancer tissues using immunohistochemistry. For both normal and prostate cancer tissues, I observed variable NKX2-5 staining in the cytoplasm as well as the nucleus (Fig 4a). The cytoplasmic staining was higher than the nuclear staining. Overall, I observed the cytoplasmic staining to be statistically less in cancers than the normal control ($p=0.034$) but not for nuclear staining ($p=0.886$). Because NKX2-5 appears to be localized in both the nucleus and cytoplasm, I wanted to know whether it is regulated by hormones. I therefore treated LNCaP cells with various ligands for 24 hr, isolated total RNA and analyzed NKX2-5 expression by RT-PCR (Fig 4b). Results showed approximately 5 fold, 8 fold, 3 fold, and 3 fold increase in NKX2-5 expression in response to testosterone, 17β-estradiol, progesterone and dexamethasome respectively, suggesting that NKX2-5 expression can be regulated by multiple hormonal signals in prostate cancer cells.



A **B**

Fig 4. A- Localization of NKX2-5 expression in human prostate tissues using immunohistochemical tissue microarrays. Expression of NKX2-5 in normal prostate (I & III) and prostate cancer (II & IV) was determined using immunohistochemical tissue microarrays as described in "Materials and Methods". Staining of cytoplasm and nucleus is detected in all tissues (original magnification, 100x). **B-** NKX2-5 expression is regulated by multiple hormones in LNCaP cells. LNCaP cells were either left untreated or

treated with the indicated ligand for 24h. Cells were harvested, total RNA was prepared and NKX2-5 expression was determined by quantitative RT-PCR analysis using iCycler and expressed relative to GAPDH to correct for variation in the amount of reverse-transcribed RNA. The data is a representative of duplicate experiments. C= control (EtOH); DHT= 5a-dihydrotestosterone; T= testosterone; Prog= progesterone; E2= 17β-estradiol; Dex= dexamethasone.

To determine whether NKX2-5 is decreased in human prostate cancer, I measured the expression of NKX2-5 in normal and neoplastic prostatic epithelium using quantitative RT-PCR analysis with mRNA from a total of 40 pairs of matched normal and tumor prostate tissue samples. I used β-actin as an endogenous mRNA control. The real-time data is presented as the ratio of NKX2-5 X 10^3/β-actin transcript for the samples analyzed (Fig. 5). The expression of NKX2-5 in both normal and cancer tissues were variable, presumably reflecting both random variability in tissue composition (i.e., epithelial content) and variable expression per cell. However, NKX2-5 expression was approximately 2 fold higher in the normal prostate tissues (5.778 $\pm$ 1.005, SEM) compared to prostate cancer (3.402 $\pm$ 0.6293, SEM, $p < 0.001$). Examination of paired normal versus cancer tissues revealed decreased NKX2-5 expression in 23 out of 38 cancer cases (60%) relative to matched normal tissues.

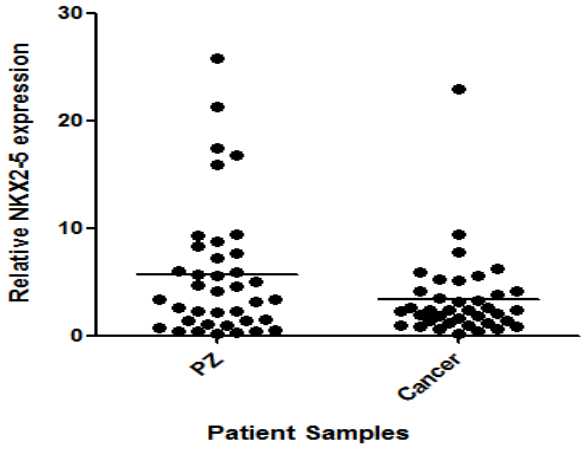
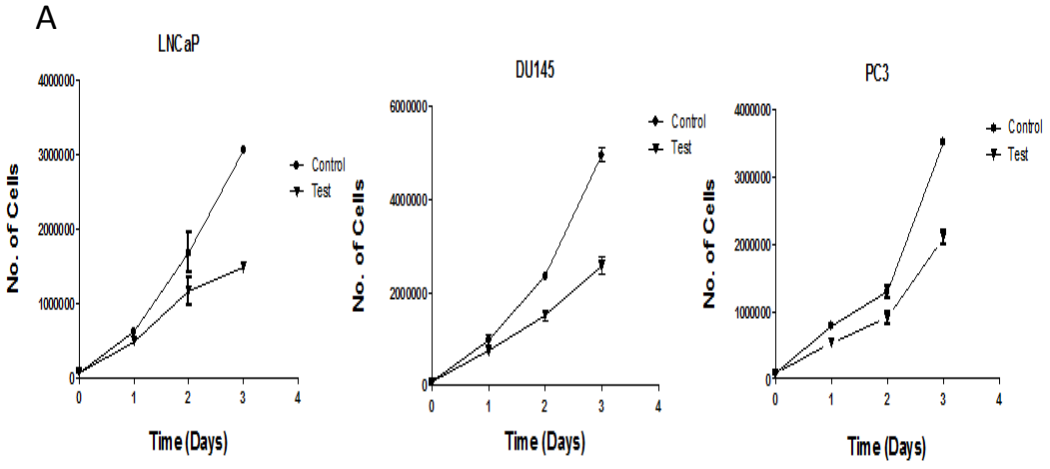


Fig 5- NKX2-5 expression in normal prostate and prostate cancer as determined by quantitative RT-PCR. NKX2-5 expression in normal prostatic peripheral zone (PZ) and cancer tissues was assessed by quantitative RT-PCR using a real-time thermal cycler (iCycler; Bio-Rad). NKX2-5 expression levels are displayed as a ratio of NKX2-5 X 10^3 to β-actin transcripts. The NKX2-5 and β-actin values were calculated from standard curves. The data are a representative of duplicate experiments. The mean expression level is indicated. The NKX2-5 expression value from cancer tissues is significantly different from the PZ tissues; $P < 0.05$ (t test) for β-actin normalization.

Effect of gain-or-loss of NKX2-5 Expression in Human Prostate Cancer Cells

To determine the biological effect of NKX2-5 expression in human prostate cancer cells, I adopted a gain-and-loss of functional approach. When prostate cancer cells were transiently transfected with pCMV6- NKX2-5 (encoding the full length of NKX2-5 sequence) I observed approximately 37%, 36% and 38% inhibition of LNCaP, DU145 and PC-3 cell proliferation respectively, over a 3 day period when compared to cells transfected with the vector only (Fig. 6a). Analysis of NKX2-5 expression in the same cells by Western blotting showed increase in NKX2-5 expression in cells transfected with the NKX2-5 expressing plasmid compared to the vector only transfection (Fig 6b). In contrast, when I endogenously knock-down NKX2-5 expression by transfecting prostate cancer cells with shRNA plasmids for human NKX2-5, I observed 89%, 50% and 60% increase in LNCaP, DU145 and PC-3 cell proliferation after 24 hours of transfection in comparison with cells transfected with scrambled oligonucleotide only (Fig. 6c). To demonstrate a consecutive down-regulation of NKX2-5, Western blot analysis was performed (Fig 6d). The results demonstrates that forced expression of NKX2-5 can significantly inhibit prostate cancer cell proliferation whereas a modest decrease in NKX2-5 protein can significantly increase prostate cancer cell proliferation clearly suggesting that NKX2-5 plays a role in prostate cancer cell proliferation.



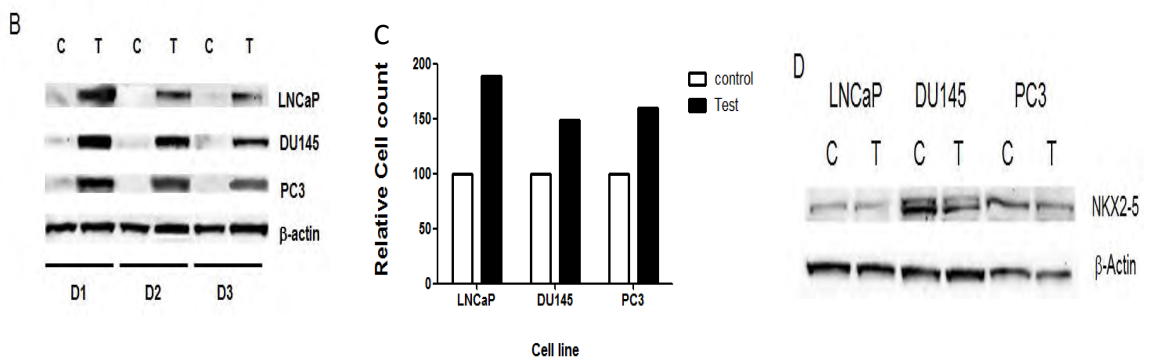


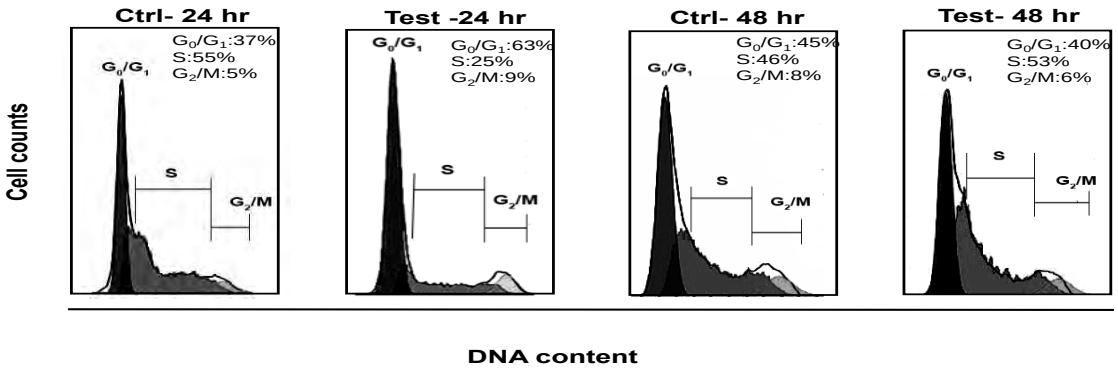
Figure 6- Function of NKX2-5 in prostate cancer cell lines. A- LNCaP, DU145, and PC3 prostate cancer cells were each transfected with NKX2-5 cDNA cloned into the mammalian expression vector pCMV6 (test) or the vector only (control). At the indicated times after transfection, cells were trypsinized and counted using a Coulter counter. All determinations were performed in triplicate, and the SD is shown. **B**- Protein extracts were collected from LNCaP, DU145, and PC3 cells 2 days post-transfection with vector control transfection (C) or NKX2-5 transfections (T) and analyzed by Western blot with either anti-NKX2-5 antibody or control anti-β-actin antibody. **C**- LNCaP, DU145, and PC3 prostate cancer cells were each transfected with shRNA plasmid to NKX2-5 (test) or scrambled shRNA (control). After 48 hr post-transfection, cells were trypsinized and counted using a Coulter counter and percentage cell proliferation in test experiments expressed relative to control (set at 100%). D- Protein extracts were collected from LNCaP, DU145, and PC3 cells 2 days post-transfection with scrambled shRNA control (C) or the NKX2-5 shRNA transfections (T) and analyzed by Western blot with either anti-NKX2-5 antibody or control anti-β-actin antibody.

NKX2-5 induces G_0/G_1 cell cycle arrest and promotes apoptosis.

I further examined how NKX2-5 might affect cell cycle progression and cell proliferation. Using transient transfection of prostate cancer cell lines with either the NKX2-5 expression plasmid or an empty vector plasmid, I compared their cell cycle distributions. The percentage of G_0/G_1 phase cells in NKX2-5 transfected cells was approximately 30% more than that in the empty vector only transfected cells, whereas NKX2-5 transfections showed less S or G_2/M phase cells than the control transfections (Fig 7a; because I observed similar responses in all 3 prostate cancer cell lines, I am only showing the response in DU145 cells). Next I determined whether NKX2-5 could affect apoptosis in prostate cancer cell lines. Using Annexin V/PI apoptotic assay, I observed about 3-fold increase apoptosis in prostate cancer cells transfected with the NKX2-5

plasmid when comparison with the vector only transfection (Fig 7b). Collectively, these findings indicate that NKX2-5 can cause cell cycle arrest and is a pro-apoptotic factor for prostate cancer cell proliferation.

7a



7b

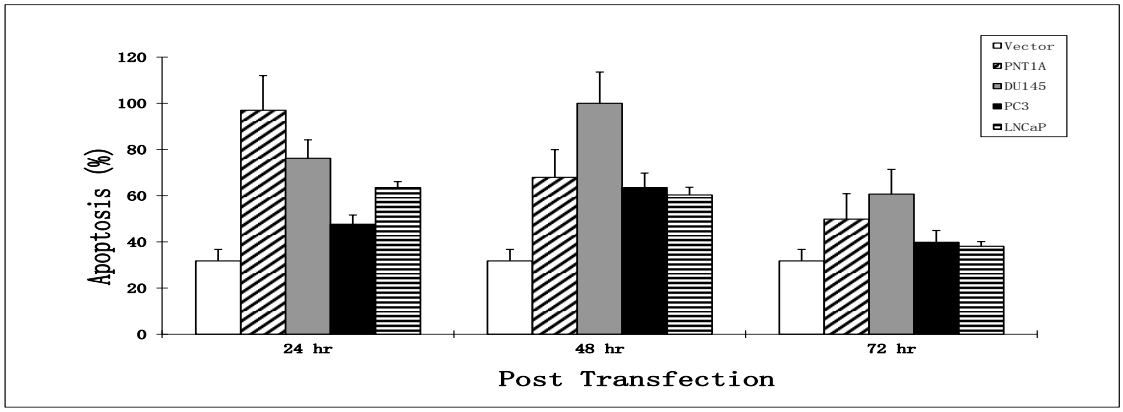


Figure 7: Nkx2-5 induces G0/G1 cell cycle arrest and apoptosis. A. Histogram of FACS cell cycle analysis of DU145 cells transfected with either scrambled shRNA (Ctrl) or shRNA to NKX2-5 (Test). Cells were harvested at 24 and 48 hr post-transfection and stained with propidium iodide. Percentage of cells in G_0/G_1, S and G_2/M phases are shown. **B.** Prostate cell lines PNT1A, DU145, PC3, and LNCaP transfected with either vector only plasmid or NKX2-5 plasmid were harvested at the indicated time points and stained with propidium iodide and apoptosis measured by Annexin-PE assay.

NKX2-5 Regulates the Expression of p53, PTEN, Histone H1, and AR in Human Prostate Cancer Cell Lines.

To investigate the involvement of NKX2-5 expression in prostate carcinogenesis, I investigated the expression levels of several target genes that are important in prostate cancer, in response to either gain-or-loss of NKX2-5 expression. Western blot analysis demonstrates the increase expression of AR, p53, PTEN, and SOD1 proteins in all prostate cell lines transfected with NKX2-5 plasmid in comparison with empty vector (Fig 8a). I did not see any significant effect of increased NKX2-5 expression on GSTP1 protein level. However, I observed an increased expression of GSTPI mRNA transcript in PNT1a and DU145 cells and somewhat reduced expression in PC3 cells by RT-PCR analysis (data not shown). In contrast, I observed reduced expression of AR, p53, PTEN, SOD1 and a modest reduction of GSTPI expression in the prostate cells transfected with NKX2-5 shRNA plasmid in comparison with scrambled oligonucleotide transfections (Fig 8b). Taken together, the data indicate an important regulatory role for NKX2-5 expression in prostate cancer cells by targeting several regulatory proteins.

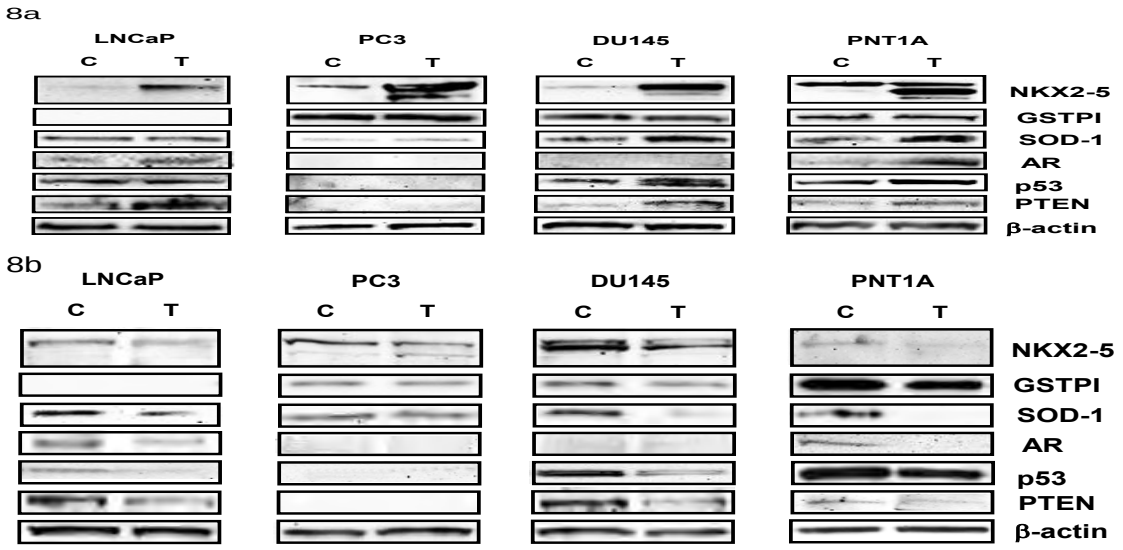


Figure 8- The effect of NKX2-5 on downstream signals. A. The prostate cell lines PNT1A, LNCaP, PC3, and DU145 were with either vector only plasmid (C) or NKX2-5 plasmid (T). **B.** The prostate cell lines were either transfected with scrambled shRNA plasmid (C) or shRNA to NKX2-5 (T). Cells were harvested 24 hr post-transfection and analyzed by Western blot with anti-NKX2-5, anti-GSTP1, anti-SOD1, anti-AR, anti-p53, anti-PTEN antibodies or control anti-β-actin antibody.

Task 5: Correlate methylation and mutation (Months 27-30)

DNA methyltransferase 3b (DNMT3b) gene variants and African American versus Caucasian prostate tissue samples.

The CpG dinucleotide marks are established and maintained by DNA methyltransferases (DNMTs) which catalyze the transfer of methyl group from S-adenosyl-methionine to cytocise bases in CpG dinucleotides. There are 4 active DNA methyltransfarases namely; DNMT1, 2, 3A and 3B. DNMT 3A and 3B carry out de novo DNA methylation changes whereas DNMT1 is considered to maintain the fidelity of DNA methylation. There are reports to suggest that polymorphism in DNMT3b may be associated with an increase in promoter methylation [16]. I therefore investigated several polymorphisms in DNMT3b genes in prostate tissue samples from AA versus Cau men. Results are shown in Table 3. Results indicate statistically significant polymorphic variants in DNMT3b in AA samples in comparison to Cau samples; however this did not correlate with the higher prevalence of DNA methylation changes observed in the AA samples. Therefore other mechanism may underlie the differences in methylation prevalence observed for the 2 ethnic groups.

Marker	Allele	Allele frequency (AA)	Allele frequency (Cau)	AA Samples	Cau Samples	p-value
rs6058869						
	C/T			64	72	0.988
	T/T	0.5	0.46	31	30	0.498
	C/C	0.5	0.54	31	40	0.509
rs6119954						
	A/G			57	36	0.0008
	G/G	0.71	0.84	62	101	0.001
	A/A	0.29	0.16	8	4	0.171
rs242908						
	C/T			6	20	0.009
	T/T	0.92	0.47	114	56	<0.00000001
	C/C	0.08	0.53	7	65	<0.00000001
rs6087990						
	C/T			32	68	0.0003
	T/T	0.36	0.53	23	34	0.361
	C/C	0.64	0.47	54	28	0.000005

Table 3: Single nucleotide polymorphisms (SNPs) detection in the promoter region of DNMT3b as determined by pyrosequencing using DNA samples derived from AA and Cau prostate tissues.

Task 6: Correlation of methylation profiles between different races and prostate tissue pathological features (Months 30-36).

A) Comprehensive Age-related DNA methylated gene profiles in AA (----) and Caucasian (-) men:

I investigated the DNA methylation status for a panel of genes in normal prostate tissue samples collected from both African American (AA) and Caucasian (Cau) men in order to determine if DNA methylation increases as a function of age for different ethnic groups. I analyzed a total of 6 genes including GSTP1, AR and RARβ2 that I and others have previously shown to be hypermethylated in prostate cancer tissue samples from Cau males [6, 17, 18]. In addition, I also analyzed SPARC, TIMP3 and NKX2-5 that I and others have identified as being hypermethylated in the gene promoter using methylated CpG island amplification (MCA) coupled with representational difference analysis (RDA) in prostate cancer cell lines [19].

I analyzed the methylation pattern in tissue punches of normal prostate collected from AA (18 samples; mean age 62.7 $\pm$ 7.2) and Cau (13 samples; mean age 62.4 $\pm$ 8.8). For each gene studied, the percentage DNA methylation at the specific promoter region

was expressed as a function of age for both the AA and Cau male samples (Fig. 9). I observed methylation of the GSTP1, RARβ2 and TIMP3 genes but this did not significantly correlate with age in both AA and Cau. The SPARC and AR genes were entirely unmethylated in the Caucasian male samples although we observed some methylation in the AA samples. Regression analysis demonstrated significantly higher methylation for TIMP3 gene ($p = 0.04$) and NKX2-5 gene ($p = 0.008$) in the AA samples when compared to the Cau samples. Furthermore, we observed a significant association between age and methylation level for NKX2-5 gene ($p = 0.03$) in the AA samples when compared to the Cau samples. The results demonstrate higher methylation of several genes in normal prostate tissue samples obtained from AA in comparison with Cau. In addition, the data indicates that for some genes the higher methylation differences may be associated with aging.

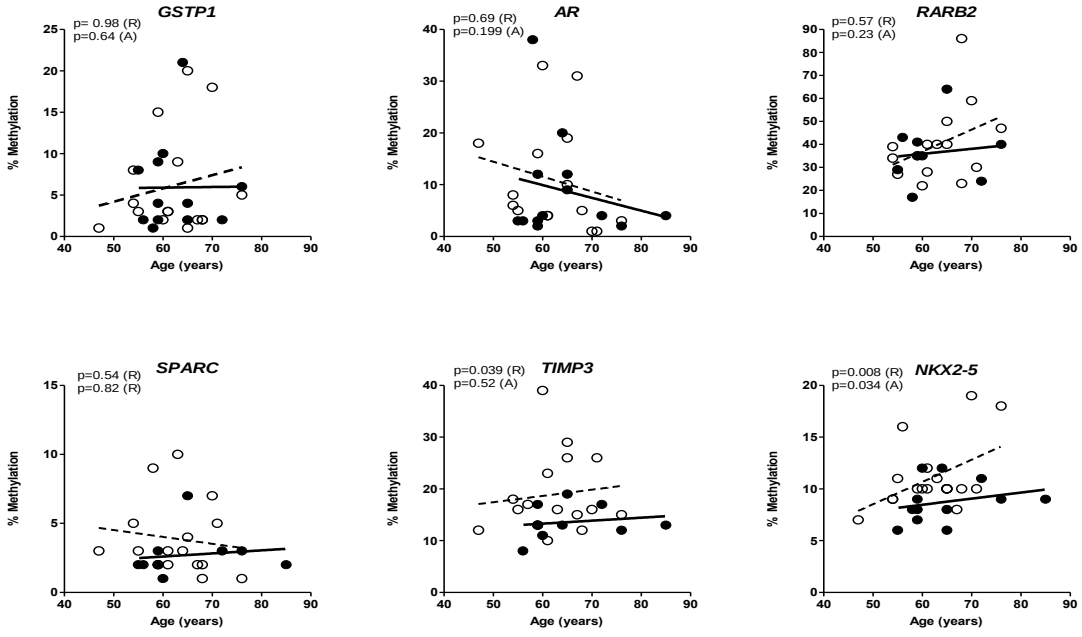


Figure 9: Age-related DNA methylated gene profiles in AA (----) and Caucasian (-) men. CpG islands for GSTP1, AR, RARβ2, SPARC, TIMP3 and NKX2-5 were analyzed in bisulfite modified genomic DNA extracted from fresh frozen tissue punches of normal prostate obtained from both AA and Cau. Eighteen samples from AA and 14 samples from Cau (age range 47-85 years) were used. Statistical analysis for the association of DNA methylation changes was determined by Race and Age. Y-axis represents the percentage of methylated cytosines in the samples as obtained from pyrosequencing. Each CpG island has a different scale range. X-axis represents age in years.

B) Quantitative DNA methylation analysis in matched benign and prostate cancer tissues from AA versus Cau men:

Next I investigated DNA methylation levels using bisulfite modified genomic DNA that were derived from radical prostatectomy specimens, obtained from both AA and Cau males with PCa in order to determine the prevalence of methylation changes in prostate cancer samples from the two groups. I analyzed the methylation status of these genes in 40 DNA samples from matched normal and prostate cancer tissue samples obtained from both AA and between 12 - 40 DNA samples from matched normal and prostate cancer tissue samples obtained from Cau males who underwent radical prostatectomy for prostate cancer (age range 52-75 years). For each gene studied, the percentage of methylation at a specific promoter was compared between the matched normal and PCa tissue samples. There was considerable variation in the percentage of CpG island methylation in the individual patient samples studied, presumably reflecting both random variability in tissue composition and variable methylation level per cell. The variable range of methylation could also reflect differences in genetic susceptibility to methylation, lifestyle or other environmental exposures (including diet), and the random nature of the methylation event. Results are shown in Figure 10.

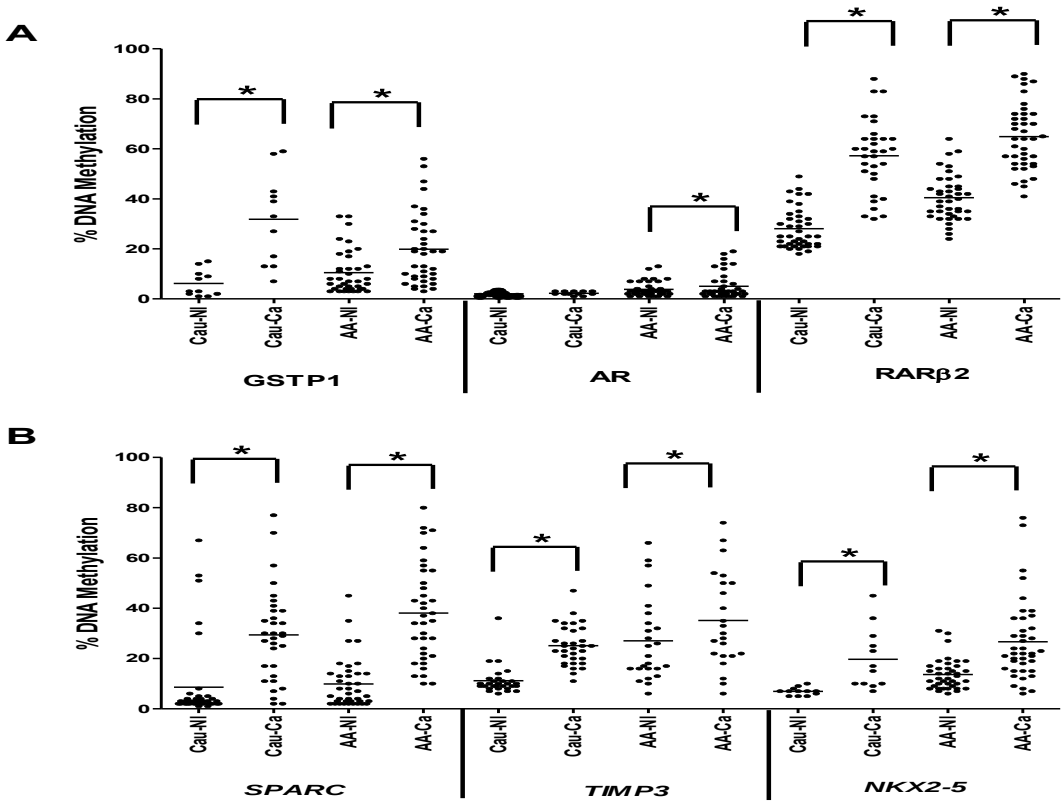


Figure 10: Quantitative DNA methylation analysis in human prostate tissues. The % DNA methylation level of promoter CpG islands were analyzed with bisulfite modified genomic DNA extracted from matched pairs of normal (Nl) and PCa (Ca) tissue samples obtained from AA and Cau cancer patients who had undergone radical prostatectomy. Y-axis represents the percentage of methylated cytosines in the samples as obtained from pyrosequencing. X-axis represents Nl and Ca tissues obtained from AA and Cau. * shows statistically significant data as determined by Mann Whitney t-test, with significance set at the level of $p< 0.05$. **A.** Quantitative methylation analysis for GSTP1, AR and RARβ2. **B.** Quantitative methylation analysis for SPARC, TIMP3 and NKX2-5.

The results showed significant hypermethylation for GSTP1 ($p = 0.0001$ for AA; $p = 0.0008$ for Cau), RARβ2 ($p < 0.001$ for AA and Cau), SPARC ($p < 0.0001$ for AA and Cau), TIMP3 ($p < 0.0001$ for AA and Cau) and NKX2-5 ($p < 0.0001$ for AA; $p = 0.003$ for Cau) in the prostate cancer tissue samples when compared with the matched normal tissue samples. The AR gene showed low prevalence of methylation in the prostate tissue samples from both AA and Cau males. Regression analysis to examine whether methylation frequency in both the normal and PCa tissue samples differ by race

showed significantly higher methylation prevalence for AR ($p = 0.006$); RARβ2 ($p < 0.0001$); SPARC ($p = 0.02$); TIMP3 ($p < 0.0001$) and NKX2-5 ($p = 0.002$) for AA in comparison with Cau samples, whereas prostate cancer and race interaction showed significance for GSTP1 ($p = 0.0069$). The data supports the observations made above that for some genes; there are higher DNA methylation levels in AA samples in comparison to Cau samples.

In addition, ROC analysis (Fig 11) was done to compare the adjacent normal prostate and matched cancer tissues in AA, Cau and both (Cau + AA) samples. The areas under the ROC curves in the Cau samples are 0.969 for GSTP1; 0.59 for AR; 0.969 for RARβ2; 0.75 for SPARC; 0.875 for TIMP3 and 0.984 for NKX2-5. The areas under the ROC curves in the AA samples are 0.811 for GSTP1; 0.531 for AR; 0.922 for RARβ2; 0.921 for SPARC; 0.668 for TIMP3 and 0.857 for NKX2-5. For the analysis in both the CAU + AA samples, the areas under the ROC curves was 0.844 for GSTP1; 0.536 for AR; 0.925 for RARβ2; 0.878 for SPARC; 0.705 for TIMP3 and 0.884 for NKX2-5. The strength of the ROC curves indicates that DNA methylation of GSTP1, RARβ2 and NKX2-5 show potential as predictive genes for prostate cancer detection in both AA and Cau male samples. On the other hand, the SPARC gene showed higher sensitivity and specificity for the AA samples in comparison with the Cau samples. Thus a large population study is needed to confirm our preliminary observations as to whether DNA methylated genes can be explored as “ethnic sensitive” biomarkers that can be used in conjunction with PSA testing for prostate cancer detection.

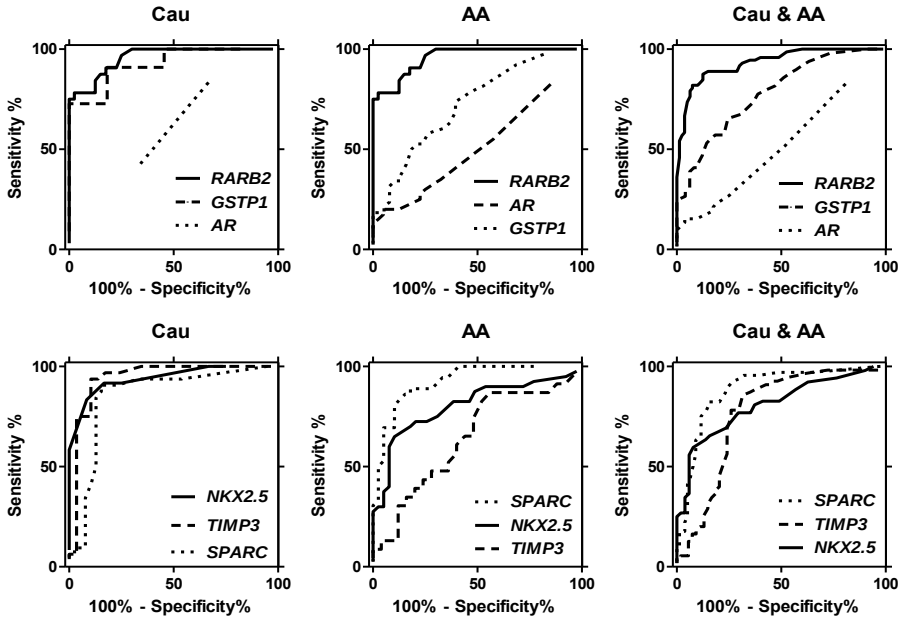


Figure 11: ROC curves for methylation analysis in prostate cancer tissue samples from AA, Cau and both (AA + Cau). The areas under the ROC curves in Cau samples are 0.969 for GSTP1; 0.59 for AR; 0.969 for RARβ2; 0.75 for SPARC; 0.875 for TIMP3 and 0.984 for NKX2-5. The areas under the ROC curves in AA samples are 0.811 for GSTP1; 0.531 for AR; 0.922 for RARβ2; 0.921 for SPARC; 0.668 for TIMP3 and 0.857 for NKX2-5. The areas under the ROC curves in both Cau + AA samples are 0.844 for GSTP1; 0.536 for AR; 0.925 for RARβ2; 0.878 for SPARC; 0.705 for TIMP3 and 0.884 for NKX2-5.

C. Study DNA methylation profiles and mutational analysis in the prostate tissues from men with high grade prostatic intraepithelial neoplasia (HGPIN) versus men invasive prostate cancer (PCa)

Some men with prostatic intraepithelial neoplasia (PIN) may never develop invasive prostate cancer, whereas other men may develop invasive prostate cancer. While DNA methylation appears to have a role in PIN as well as invasive prostate cancers, it is possible that methylation in some key regulatory genes may accompany the transformation from PIN to invasive adenocarcinoma. By comparing gene methylation profile of prostate tissues from men with PIN versus men with prostate cancer, it may be possible to identify key regulatory gene(s) that are associated with the transition from PIN to prostate cancer. To achieve this, I have used genome-wide methylation analysis

based on the Infinium Human Methylation 450 BeadChip to interrogate more than 450,000 methylation sites per sample at single nucleotide resolution.

In principle, the Methylation 450 array utilizes Illumina's proven SNP typing chemistry - Infinium®- following bisulphite conversion to interrogate in excess of 450,000 methylated sites at single base resolution. In brief, 1 μl of bisulfite-converted DNA was used for hybridization on Infinium Human Methylation 450 BeadChip, following the Illumina Infinium HD Methylation protocol. This involved a whole genome amplification step followed by enzymatic end-point fragmentation, precipitation and resuspension. The resuspended samples were hybridized on Human Methylation 450 BeadChips. Then unhybridized and non-specifically hybridized DNA was washed away, followed by a single nucleotide extension using the hybridized bisulfite-treated DNA as a template. The nucleotides incorporated were labeled with biotin (ddCTP and ddGTP) and 2, 4-dinitrophenol (DNP) (ddATP and ddTTP). After the single base extension, repeated rounds of staining were performed with a combination of antibodies that differentiated DNP and biotin by fixing them different fluorophores. Finally the BeadChip was washed and scanned.

Scanning beadchips The Illumina HiScan SQ scanner is a two-color laser (532 nm/660 nm) fluorescent scanner with 0.375 μm spatial resolution capable of exciting the fluorophores generated during the staining step of the protocol. The intensities of the images were extracted using Genome Studio (2010.3) Methylation module (1.8.5) software. The methylation score for each CpG was represented as a Beta value according to the fluorescent intensity ratio. Beta values may take any value between 0 (nonmethylated) and 1 (completely methylated) as continuous variable.

Primary Data analyses Quality control checked as briefly, samples were monitored for coverage (fraction of CpGs with detectable intensity values above background) and bisulphite conversion efficiency (BSCE) using the controls provided on the Illumina Beadchip. GenomeStudio normalizes data using different internal controls that are present on the Human Methylation 450 BeadChip. It also normalizes data depending on internal background proves. We did apply transformation to raw data.

Statistical Analysis. Initial array results were visualised using Illumina® BeadStudio 3.2. All other computations and statistical analyses were performed using Partek Genome Studio. CpG methylation differences were considered significant above a cut-off of a 0.6-fold change in the β value, unless specifically indicated otherwise. We used p value $< 10^{-3}$ as cut off to consider significant change. Results of methylation pattern is presented as heatmap in Fig 12.

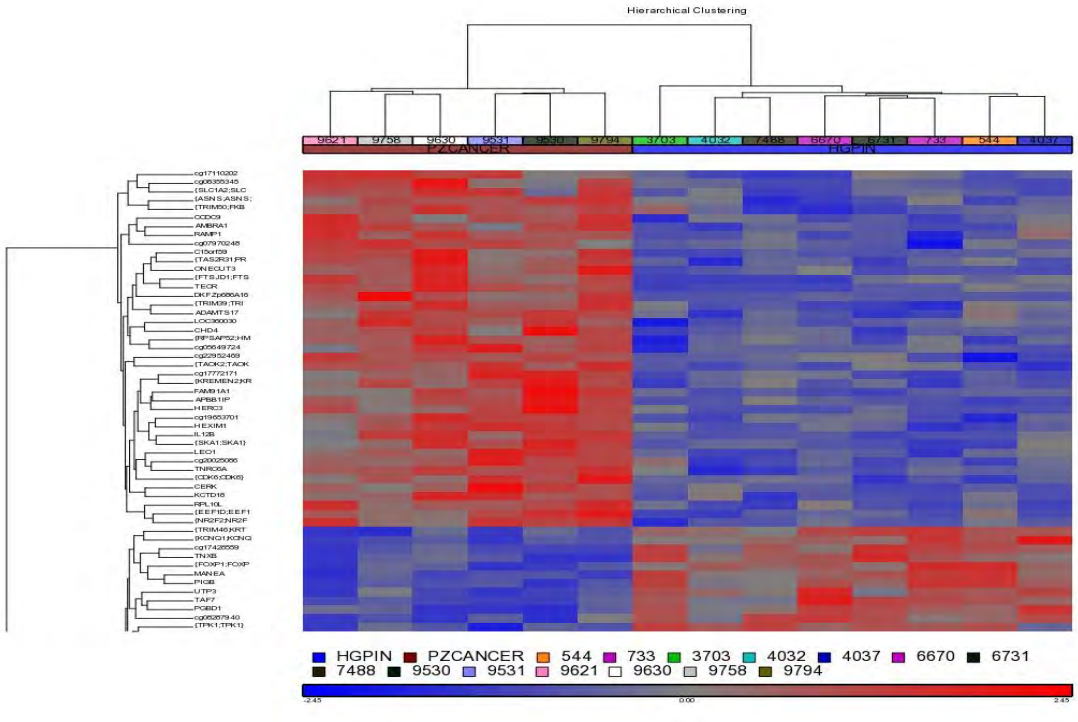


Figure 12- Heatmap analysis of methylation patterns among HGPIN and PCa samples.

Overall, I have identified more than 600 unique CpG loci that show significant differences between HGPIN and prostate cancer based on beta value (% methylation of CpG island). An on-going study is to use bioinformatics tools in order to identify important epigenetic pathways that may contribute to transformation from pre-invasive to invasive prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS

- I have identified several novel methylated genes using methylated CpG island amplification (MCA) technique coupled with CpG promoter microarray in the human prostate cancer line LNCaP.
- I have shown differential methylation of these genes in a panel of 21-cell lines derived from prostate, breast, colorectal, leukemia and liver tissues using pyrosequencing as a quantitative approach to measure methylation status. In addition, I have demonstrated in prostate tissue samples that compared with the methylation data from normal prostate tissues, there is significantly higher methylation in prostate cancer tissues for FOXN4, TIMP3, RPRM, SPARC and CYP27B1. On the other hand, CHD11 and PAX9 genes do not show a significant difference in DNA methylation between the normal and matched prostate cancer tissues.
- I have demonstrated higher methylation of several genes in prostate tissues samples from African American (AA) in comparison with Caucasian (Cau) men. Statistical analysis showed significantly higher methylation in the prostate cancer tissue samples in comparison with matched normal samples for GSTP1 ($p = 0.0001$ for AA; $p = 0.0008$ for Cau), RARβ2 ($p < 0.001$ for AA and Cau), SPARC

($p < 0.0001$ for AA and Cau), TIMP3 ($p < 0.0001$ for AA and Cau) and NKX2-5 ($p < 0.0001$ for AA; $p = 0.003$ for Cau). Overall, I observed significant differences ($p < 0.05$) in the methylation level for all genes (except GSTP1) in the AA samples in comparison to the Cau samples. Furthermore, regression analysis revealed significantly higher methylation for NKX2-5 ($p = 0.008$) and TIMP3 ($p = 0.039$) in normal prostate tissue samples from AA in comparison with Cau, and a statistically significant association of methylation with age for NKX2-5 ($p = 0.03$) in AA in comparison with Cau. Results suggests that the higher DNA methylation changes identified in AA samples may potentially contribute to the racial differences that are observed in prostate cancer pathogenesis.

- I have investigated several genetic variants in DNA methyltransferase 3b (DNMT3b) gene as a potential mechanism for the association of increase promoter methylation in prostate tissue samples from AA in comparison to Cau men. I did not see any significant association polymorphisms in DNMT3b with higher DNA methylation changes in AA samples.
- I have carried out studies investigating the biological activity of NKX2-5 in prostate cell lines. I have carried out gain-and-loss functional studies of NKX2-5 in prostate cancer cell lines and validated expression at the RNA transcript level using quantitative RT-PCR. Over-expression of NKX2-5 was detrimental to prostate cancer cell proliferation as evidenced by significant inhibition of prostate cancer cell proliferation in comparison to vector only control and this is due to cell arrest in G_o/G_1 phase and increase apoptosis. In contract, successful knockdown of NKX2-5 by shRNA transfection increased prostate cancer cell

proliferation. Western blot analysis of NKX2-5 signaling demonstrates that NKX2-5 plays a key regulatory role in the expression of several important genes including p53, PTEN, and the androgen receptor. My observations indicate that NKX2-5 is a potential tumor suppressor gene that is frequently inactivated in prostate cancer.

- I have used genome-wide methylation analysis to reveal novel methylated patterns among pre-invasive and cancerous lesions of the prostate using HGPIN and prostate cancer tissue samples from individual prostate cancer patients. To accomplish this, I have used Infinium Human Methylation 450 BeadChip to interrogate more than 450,000 methylation sites per sample at single nucleotide resolution. Overall, there are more than 600 unique CpG loci that shows significant differences between HGPIN and prostate cancer based on beta value (% methylation of CpG island). An on-going study is to use bioinformatics tools in order to identify important epigenetic pathways that may contribute to transformation from pre-invasive to invasive prostate cancer.

REPORTABLE OUTCOMES

Abstract presentation

- AACR Annual Meeting- Differential DNA methylation profiles reveals novel pathways in prostate carcinogenesis (2009). Denver, CO.
- AACR Annual Meeting- Identification of differentially Methylated Genes in Normal Prostate Tissues from African-American and Caucasian Men (2010). Washington D.C

- ❖ AACR Annual Meeting-NKX2-5, a potential tumor suppressor gene is down-regulated in prostate cancer (2011). Orlando, FL.
- ❖ DoD IMPaCT Meeting- The role of epigenetic DNA methylation changes in Aging and prostate cancer disparity (2011). Orlando, FL.

Invited Speaker

- ➢ 2011 Speaker, Clark Atlanta University, Symposium on Prostate Cancer
- ➢ 2011 Speaker, Department of Defense IMPaCT Meeting
- ➢ 2010 Speaker, Johns Hopkins School of Public Health LunchLearnLink.
- ➢ 2010 Speaker, Howard University Department of Biochemistry Seminar
- ➢ 2009 Speaker, Howard University Department of Pharmacy Seminar
- ➢ 2008 Speaker, NCI Cancer Health Disparities Summit

Bibliography

- Darimipourain M, Wang S, Ittmann M, **Kwabi-Addo B** (2011).Transcriptional and post-transcriptional regulation of Sprouty1, a Receptor Tyrosine Kinase Inhibitor in Prostate Cancer (In press; Prostate Cancer and Prostatic Diseases).
- **Kwabi-Addo B**, Wang S, Chung W, Jelinek J, Patierno SR, Wang BD, Andrawis R, Lee NH, Apprey V, Issa JP, Ittmann M (2010). Identification of differentially methylated genes in normal prostate tissues from African American and Caucasian Men. Clin. Cancer Res. 16: 3539-3547.
- **Kwabi-Addo B**, Ren C, Ittmann M (2009). DNA methylation and aberrant expression of Sprouty1 in human prostate cancer. Epigenetics. 4:54-61.

Manuscript submitted

- Daremipouran M, Wang S, Ellis A, Bamji Z, Abajomi B, Ittmann M, **Kwabi-Addo B** (2011). NKX2-5, a Potential Tumor Suppressor Gene is Down-Regulated in Prostate Cancer. (Under Review; PLoS ONE)

Manuscript in preparation

- Suer F, Wang S, Wang BD, Andrawis R, Lee NH, Ittmann M, Devaney J, **Kwabi-Addo B** (2011). Genome-wide methylation analysis reveals novel methylated patterns among pre-invasive and cancerous lesions of the prostate.

Funding applied for based on work supported by this award

Some of my preliminary results from studies described in this proposal have demonstrated that there exist differential methylation changes of several key regulatory genes in prostate tissues from African American in comparison with their Caucasian male counterpart, to suggest that epigenetic changes could potentially be the link between environmental and genetic predisposition to prostate cancer. Based on these results I have applied for the following:

- In order to explore this possibility, Dr. Srinivasan Yegnasubramanian (Johns Hopkins University, Baltimore MD) and I have been awarded a new DoD grant: Health Disparity Research Award- Qualified Collaborator Option from 8/15/2011-8/14/2014 to carry out the proposed study entitled “Global Epigenetic Changes may Underlie Ethnic Differences and Susceptibility to Prostate Cancer”.

- I have also applied for NIH funding (R21) on a proposal entitled "Analysis of the Metabolome, Transcriptome, and DNA methylation pattern of Prostate Tumors in African-American (AA) and European-American (EA) Patients for Biomarker Discovery" (Not funded).

Research opportunity based on experience/training supported by this award

- Through the continuous research grant support from DoD, I have been promoted to an Associate Professor position on tenure track level in the Department of Biochemistry and Molecular Biology at Howard University. This means additional responsibility as a lecturer but Howard University is committed to my 70% effort towards laboratory research.
- I have also had the opportunity to visit Children"s Hospital, Washington D.C. to learn and use illumina microarray platform for genome-wide methylation studies and bioinformatics tools to study gene pathways.

List of personnel support from grant:

Mr. Songping Wang- Full time Research Associate (100% salary supported from grants)

Summer Students (Summer „09-11)- 1 Part time summer student/per year supported from grant.

CONCLUSION

I have made substantial contribution from studies carried out here in understanding the role of DNA methylation changes in prostate cancer: My manuscript in Clinical Cancer Research (Clin. Cancer Res. 16:3539-47) is the first to analyze the

methylation status of several regulatory genes in normal as well as matched pairs of normal and PCa tissue samples from both AA and Cau males. Overall, I observed significant methylation prevalence in the PCa tissue samples from AA in comparison with Cau. The Cau cancer samples used in this study showed slightly higher Gleason score and similar pathological staging when compared to the AA samples. Thus the higher prevalence of methylation seen in the AA cancer samples is not simply a reflective of differences in disease aggressiveness or stage between the two groups. In addition, regression analysis revealed significantly higher methylation for some genes in the normal prostate tissue samples from AA in comparison with Cau. Of the 6 genes that I analyzed in the normal prostate tissue samples, methylation of NKX2-5 also showed a significant association with age in AA in comparison with Cau. My data suggests that not only is the methylation of NKX2-5 suitable as a marker for PCa detection, but it may also have increased sensitivity for detecting PCa in AA.

Furthermore, ROC analysis shows differential predictive potential of DNA methylation of GSTP1, RARβ2, SPARC, TIMP3 and NKX2-5 genes for PCa detection in African American and Caucasian samples. Thus, a larger PCa population size can confirm our observations and determine if the methylation status of these genes can provide a reliable and/or perhaps "ethnic sensitive" index for the detection of PCa.

Genome wide methylation studies have also revealed novel methylated patterns among pre-invasive and cancerous lesions of the prostate. I am in the process of identifying key pathways where epigenetic alterations may be affected to contribute to the transformation from pre-invasive to invasive prostate cancer. This would potentially help in understanding the important steps involved in neoplastic transformation.

'So what section"

For human prostate cancer, there is abundant evidence to suggest that somatic epigenetic alterations such as DNA methylation may appear earlier during cancer development than genetic changes, as well as more commonly and consistently. Although it is not entirely clear why epigenetic patterns are corrupted in human cancers there are possible explanations and findings from many investigations to suggest various factors that may contribute to abnormal epigenetic changes in prostate pathogenesis. There is growing evidence to suggest that epigenetic DNA methylation changes affects gene expression in an age-dependent and tissue specific manner. I have recently demonstrated that indeed CpG hypermethylation increases with age in the normal human prostate tissues [20]. Such age-dependent DNA methylation changes can alter cell physiology and possibly, predispose prostate cells to neoplastic transformation. Also in many cases, aberrant methylation precedes full-blown malignancy and can often be found in non-cancerous tissues; in the prostate, hypermethylation of the GSTP1 CpG has been detected in PIA lesions [21]. Work carried out by De Marzo et al., suggests that exposure to environmental factors such as infectious agents and dietary carcinogens, and hormonal imbalances lead to injury of the prostate and subsequently the development of chronic or recurrent inflammation [22]. Other studies have shown that known forms of endogenous DNA damage can cause either hypermethylation or hypomethylation. Inflammation induced 5-halogenated cytosine damage products, including 5-chlorocytosine, mimic 5-methylcytocine and induce inappropriate DNA methylation within the CpG sites, suggesting that genome-damaging stresses due to both endogenous and exogenous

carcinogens can cause heritable changes in cytosine methylation patterns resulting in tumor formation [23].

The current literature suggests a complex mechanism of epigenetic regulation in prostate cancer including DNA methylaytion changes that can lead to either gene silencing or the activation of key regulatory genes in the disease pathway. Underlying this aberrant DNA methylation is the accumulating body of data hinting that normal prostate cells may be subjected to a relentless barrage of genome-damaging stresses due to both exogenous and endogenous carcinogens, with damage accumulating over time and age. Thus aberrant epigenetic DNA methylation changes may represent the integration of environmental or lifestyle exposures and genetic predisposition to prostate cancer. Such events may differ between individuals belonging the same ethnic group or individuals belonging to different ethnic groups.

Continuing studies to elucidate genome-wide DNA methylation changes in prostate tissues from different ethnic groups would contribute to our understanding of the molecular mechanisms underlying prostate cancer disparity and potentially lead to the identification of "ethnic sensitive" biomarkers for early disease detection and for distinguishing indolent from aggressive prostate cancer as well as identification of potential novel epigenetic therapies for prostate cancer treatment.

Reference List

[1] Hsing AW, Tsao L, Devesa SS. International trends and patterns of prostate cancer incidence and mortality. Int J Cancer 2000;85:60-7.

[2] Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003
65. CA Cancer J Clin 2003;53:5-26.

[3] Freedland SJ, Isaacs WB. Explaining racial differences in prostate cancer in the United States: sociology or biology? Prostate 2005;62:243-52.

[4] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57-70.

[5] Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042-54.

[6] Kwabi-Addo B, Chung W, Shen L, et al. Age-related DNA methylation changes in normal human prostate tissues. Clin Cancer Res 2007;13:3796-802.

[7] Hmadcha A, Bedoya FJ, Sobrino F, Pintado E. Methylation-dependent gene silencing induced by interleukin 1beta via nitric oxide production. J Exp Med 1999;190:1595-604.

[8] Feinberg AP, Tycko B. The history of cancer epigenetics. Nat Rev Cancer 2004;4:143-53.

[9] Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415-28.

[10] Nelson WG, De Marzo AM, Isaacs WB. Prostate cancer. N Engl J Med 2003;349:366-81.

[11] Bastian PJ, Yegnasubramanian S, Palapattu GS, et al. Molecular biomarker in prostate cancer: the role of CpG island hypermethylation. Eur Urol 2004;46:698-708.

[12] Ahmed H. Promoter Methylation in Prostate Cancer and its Application for the Early Detection of Prostate Cancer Using Serum and Urine Samples. Biomark Cancer 2010;2010:17-33.

[13] Li LC, Okino ST, Dahiya R. DNA methylation in prostate cancer. Biochim Biophys Acta 2004;1704:87-102.

[14] Toyota M, Ho C, Ahuja N, et al. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. Cancer Res 1999;59:2307-12.

[15] Kwabi-Addo B, Wang S, Chung W, et al. Identification of differentially methylated genes in normal prostate tissues from African American and Caucasian men. Clin Cancer Res 2010;16:3539-47.

[16] Singal R, Das PM, Manoharan M, Reis IM, Schlesselman JJ. Polymorphisms in the DNA methyltransferase 3b gene and prostate cancer risk
104. Oncol Rep 2005;14:569-73.

[17] Ellinger J, Bastian PJ, Jurgan T, et al. CpG island hypermethylation at multiple gene sites in diagnosis and prognosis of prostate cancer
12. Urology 2008;71:161-7.

[18] Tokumaru Y, Harden SV, Sun DI, Yamashita K, Epstein JI, Sidransky D. Optimal use of a panel of methylation markers with GSTP1 hypermethylation in the diagnosis of prostate adenocarcinoma
3. Clin Cancer Res 2004;10:5518-22.

[19] Chung W, Kwabi-Addo B, Ittmann M, et al. Identification of novel tumor markers in prostate, colon and breast cancer by unbiased methylation profiling
3. PLoS One 2008;3:e2079.

[20] Kwabi-Addo B, Chung W, Shen L, et al. Age-related DNA methylation changes in normal human prostate tissues. Clin Cancer Res 2007;13:3796-802.

[21] Nakayama M, Bennett CJ, Hicks JL, et al. Hypermethylation of the human glutathione S-transferase-pi gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using laser-capture microdissection
24. Am J Pathol 2003;163:923-33.

[22] De Marzo AM, Platz EA, Sutcliffe S, et al. Inflammation in prostate carcinogenesis
49. Nat Rev Cancer 2007;7:256-69.

[23] Valinluck V, Sowers LC. Endogenous cytosine damage products alter the site selectivity of human DNA maintenance methyltransferase DNMT1
21. Cancer Res 2007;67:946-50.

AACR American Association for Cancer Research 102nd ANNUAL M
April 2-6, 2011 • Orange County Convention Center

Abstract Number: 3033

Presentation Title: NKX2-5, a potential tumor suppressor gene is down-regulated in prostate cancer

Author Block: Mohammad Daremipouran, Song Ping Wang, Alexander Ellis, Zebalda Bamji, Bernard Kwabi-Addo. Howard University, Washington, DC

Abstract Body: Background: Prostate cancer (PCa) is a common malignancy and a leading cause of cancer deaths among men in the United States. Abundant evidence has accumulated to suggest that epigenetic DNA methylation changes may appear earlier during PCa development than genetic changes, as well as more commonly and consistently suggesting that DNA methylated genes can be explored as DNA-based biomarker for PCa disease detection. Recently, we have identified NKX2-5 as a novel that is hypermethylated in prostate cancer. However, there is little information about the biological significance of this gene in prostate carcinogenesis. We hypothesize that NKX2-5 is a potential tumor suppressor gene that is frequently inactivated in prostate cancer. Methods: We carried out gain-and-loss functional studies of NKX2-5 in prostate cancer cell lines and validated expression at the RNA transcript level using quantitative RT-PCR. Protein expression was analyzed by western blotting and cell cycle analysis investigated by flow cytometer. Results: Over-expression of NKX2-5 was detrimental to prostate cancer cell proliferation as evidenced by significant inhibition of prostate cancer cell proliferation in comparison to control (vector only transfection) and this was due to cell arrest in Go/G1 phase and increase apoptosis. In contract, successful knockdown of NKX2-5 by shRNA transfection increased prostate cancer cell proliferation. Western blot analysis demonstrated that NKX2-5 plays a key regulatory role in the expression of several genes including p53, PTEN, Histone H1 and the androgen receptor. Conclusion: Our observation suggests that NKX2-5, a member of the homeobox gene family of plays an important tumor suppressor activity in prostate carcinogenesis. Because this gene plays important role in several signal transduction pathways, this gene can be exploited as potential biomarker for the early detection of prostate cancer and could be an attractive target to explore for drug investigation or gene therapies of prostate cancer.

ONLINE PROCEEDINGS / ITINERARY PLANNER
AACR 101st ANNUAL MEETING 2010
April 17-21, 2010 • Walter E. Washington Convention Center • Washington, DC

Presentation Abstract

Add to Itinerary

Print

Abstract Number:	167
Presentation Title:	Identification of differentially methylated genes in normal prostate tissues from African American and Caucasian men
Presentation Time:	Sunday, Apr 18, 2010, 2:00 PM - 5:00 PM
Location:	Exhibit Hall A-C, Poster Section 5
Poster Section:	5
Poster Board Number:	27
Author Block:	Bernard Kwabi-Addo[1], Songping Wang[1], Norman H. Lee[2], Marcos R. Estecio[3], Jaroslav Jelinek[3], Woonbok Chung[3], Michael M. Ittmann[4]. [1]Howard University, Washington, DC; [2]George Washington University, Washington, DC; [3]University of Texas M.D. Anderson Cancer Ctr, Houston, TX; [4]Baylor College of Medicine, Houston, TX
Abstract Body:	**BACKGROUND.** Prostate cancer (PCa) is a common malignancy and a leading cause of cancer deaths among men in the United States. African American men have both a higher incidence and significantly higher mortality rates than Caucasian men. Abundant evidence has accumulated to suggest that epigenetic DNA methylation changes may appear earlier during PCa development than genetic changes, as well as more commonly and consistently. Most studies have emphasized DNA hypermethylation as an important mechanism for inactivation of key regulatory genes in prostate cancers. Thus methylated genes can serve as biomarkers for the detection of cancer from clinical specimens such as tissue biopsies or body fluids. However, very few studies have investigated differences in DNA methylation pattern in different ethnic groups or their application as ethnic sensitive biomarkers for disease detection. We hypothesize that differential methylation patterns maybe associated with ethnicity and could potentially contribute to the incidence and mortality of prostate cancer. **METHODS.** We have identified several genes to be differentially methylated in human prostate cancer cell line using methylated CpG island amplification coupled with CpG promoter microarray. Pyrosequencing was used to quantitatively measure the methylation levels of CDH11, FOXN4, PAX9, TIMP3, RPRM, SPARC, TCF3,CYP27B1, RARβ2, AR, GSTP1, NKX2-5, RASSF1A and TIMP3 genes in prostate cell lines, matched pairs of benign and prostate cancer tissues from individual prostate cancer patients, and normal prostate biopsies from African American and Caucasian men. Real time PCR was used to determine gene expression at the RNA transcript level. **RESULTS.** DNA hypermethylation was observed for NKX2-5, FOXN4, TIMP3, RPRM, TCF, SPARC, GSTP1, RASSF1A, RARβ2 and SPARC in prostate cancer cells and prostate cancer tissues samples. Higher frequency of

methylation was observed for several genes including NKX2-5, GSTP1, RASSF1A, and RARβ2 genes in punch biopsies of normal prostate samples obtained from African American men in comparison to samples from Caucasian men. **CONCLUSION.** Our observations suggest a genome wide differential methylation patterns in prostate tissue samples obtained from African American men versus Caucasian men. Such alterations could account for some of the biological differences underlying prostate cancer disparity.

American Association for Cancer Research
www.aacr.org
615 Chestnut St.
17th Floor
Philadelphia, PA 19106

Abstract Number: 5191

Session Title: DNA Methylation 2

Presentation Title: **Differential DNA methylation profiles reveals novel pathways in prostate carcinogenesis**

Presentation Start/End Time: Wednesday, Apr 22, 2009, 8:00 AM -12:00 PM

Location: Hall B-F, Poster Section 19

Poster Section: 19

Poster Board Number: 20

Author Block: *Bernard Kwabi-Addo*, *Songping Wang*, *Jaroslov Jelinek*, *Jean Pierre Issa*, *Michael Ittmann*. Howard Univ. Cancer Ctr., Washington, DC, MD Anderson Cancer, Houston, TX, Baylor College of Medicine, Houston, TX

The progressive acquisition of genomic alterations and epigenetic defects including changes in cytosine methylation patterns is a defining feature of all human cancers including prostate cancer. For human prostate cancer, abundant evidence has accumulated to suggest that somatic epigenetic alterations may appear earlier during cancer development as well as more commonly and consistently.

To identify novel CpG islands that are aberrantly methylated in prostate cancer, we used methylated CpG island amplification (MCA) couple with promoter CpG microarray analysis in the prostate cancer cell line, LNCaP. We identified more than 300 differentially hypermethylated loci of which approximately 50 were unique promoter associated CpG islands. We identified 21 clones to correspond to hypermethylated genes that maybe important in prostate carcinogenesis including Pax9, Reprimo, Cadherin 11, Notch1, Osteonection, netrin 4, Foxn4, Timp3, and Flt1. Similarly, we identified about 374 differentially hypomethylated loci in the LNCaP cells. However, 349 of these probes mapped to repetitive elements and only 25 loci were unique promoter associated CpG islands including the transcription factors TCF3 and ZNF306, Cadherin 12 and PTPRN2. The gene expression patterns of these genes were verified in prostate cell lines and tissue samples. Hypermethylation resulted in down-regulation of several genes including Osteonectin and Cadherin 11 in the prostate cancer cell lines and tissues in comparison to normal prostatic primary cells and benign prostate tissues. Hypomethylation on the other hand, led to the activation of several genes including Cadherin 12 and TCF3.

These data suggests epigenetic modulation of several genes and transcription factors in prostate cancer. Cumulative effect of these epigenetic changes may not only ensure cancer of the prostate but may also contribute to the metastasis of prostate cancer cells to other tissue sites.

2009 AACR Annual Meeting
April 18-22, 2009
Denver, CO

Citation Format: {Authors}. {Abstract title} [abstract]. In: Proceedings of the 100th Annual Meeting of the American Association for Cancer Research; 2009 Apr 18-22; Denver, CO. Philadelphia (PA): AACR; 2009. Abstract nr {Abstract number}

Disclosure Information for CME-Designated Sessions

25th Jun 2011

RE: Manuscript PCAN-11-0108R, Transcriptional and post-transcriptional regulation of Sprouty1, a Receptor Tyrosine Kinase Inhibitor in Prostate Cancer

Dear Dr Kwabi-Addo,

I am pleased to inform you that your manuscript has been accepted for publication.

If you have chosen to make your manuscript open-access, please download, print, sign and return the following License to Publish forms using the following links:

http://mts-pcan.nature.com/letters/PCANOpen_LTP.pdf (License to Publish) http://mts-pcan.nature.com/letters/pcan_LTP_OpenAccess.pdf (Payment Form)

If you have NOT chosen to make your manuscript open-access, please use this License to Publish form:

http://mts-pcan.nature.com/letters/pcan_copyright.pdf

If your manuscript contains color figures and you would like them printed in color, please indicate this in the "Charges" information tab.

The production editor will notify you when the manuscript has published online. This online publication will serve as your manuscript's official date of publication.

Your manuscript will be processed simultaneously by Nature Publishing Group for print publication. Manuscripts in the printed and electronic editions of Prostate Cancer and Prostatic Diseases will be copy edited, and you will be provided page proofs prior to publication.

If you have any questions, please do not hesitate to call or email me the editorial office. Thank you for your contribution to Prostate Cancer and Prostatic Diseases.

Sincerely,

Stephen Freedland
Editor
Prostate Cancer and Prostatic Diseases

****

As your manuscript has been accepted for publication by Prostate Cancer and Prostatic Diseases (PCAN) you may be eligible to participate in the PEER project. If you are based in the European Union, Nature Publishing Group may deposit your accepted manuscript on your behalf, in one of the participating PEER repositories. There will be an embargo period of 6 months from the date of publication of the journal article for the public release of your accepted manuscript. You need take no further action.

For further information please visit the PEER project website helpdesk: http://www.peerproject.eu/
**********If you have not already done so, we strongly recommend that you upload the step-by-step protocols used in this manuscript to the Protocol Exchange. Protocol Exchange is an open online resource that allows researchers to share their detailed experimental know-how. All uploaded protocols are made freely available, assigned DOIs for ease of citation and fully searchable through nature.com. Protocols can be linked to any publications in which they are used and will be linked to from your article. You can also establish a dedicated page to collect all your lab Protocols. By uploading your Protocols to Protocol Exchange, you are enabling researchers to more readily reproduce or adapt the methodology you use, as well as increasing the visibility of your protocols and papers. Upload your Protocols at www.nature.com/protocolexchange/. Further information can be found at www.nature.com/protocolexchange/abou
t .

PCAN - This email has been sent through the NPG Manuscript Tracking System NY-610A-NPG&MTS

Transcriptional and post-transcriptional regulation of Sprouty1, a Receptor Tyrosine Kinase Inhibitor in Prostate Cancer

Mohammad Darimipourain[1], Songping Wang[1], Michael Ittmann[2] and Bernard Kwabi-Addo[1]*.

[1]*Howard University Cancer Center, 2041 Georgia Ave, N.W. Washington D.C. 20060.*

[2]*Department of Pathology, Baylor College of Medicine, 1 Baylor Plaza, Houston TX 77030 USA.*

RUNNING TITLE: Transcriptional and post-transcriptional regulation of Sprouty1

KEY WORDS: Spry1; transcription; microRNAs, prostate cancer

*Correspondence:

Bernard Kwabi-Addo, PhD,

Howard University Cancer Center

2041 Georgia Ave. N.W. Washington D. C. 20060

Tel: 202 806 9149

Fax: 202 667 1686

E-mail: bkwabi-addo@howard.edu

Abstract

Sprouty1 (Spry1) is a negative regulator of fibroblast growth factor signaling with a potential tumor suppressor function in prostate cancer (PCa). Spry1 is down-regulated in human PCa and Spry1 expression can markedly inhibit PCa proliferation *in vitro*. We have reported DNA methylation as a mechanism for controlling *Spry*1 expression. However, promoter methylation does not seem to explain gene silencing in all prostate cancer cases studied to suggest other mechanisms of gene inactivation such as alterations in trans-acting factors and/or post-transcriptional activity may be responsible for the decreased expression in those cases. Binding sites for Wilm's tumor (WT1) transcription factors: EGR1, EGR3 and WTE are highly conserved between the mouse and human *Spry*1 promoter regions, suggesting an evolutionary conserved mechanism(s) involving WT1 and EGR in *Spry*1 regulation. *Spry*1 mRNA contains multiple microRNA (miRNA) binding sites in its 3'UTR region suggesting post-transcriptional control. We demonstrate that *Spry*1 is a target for miR-21 mediated gene silencing. miRNA-based therapeutic approaches to treat cancer are emerging. Spry1 is highly regulated by miRNAs and could potentially be an excellent candidate for such approaches.

INTRODUCTION

Prostate cancer (PCa) is the second most common malignancy and the second leading cause of cancer deaths in men in the United States. There is abundant evidence to indicate that inappropriate activation of fibroblast growth factor receptor (FGFR) signaling plays a critical role in the initiation and progression of prostate cancer (for review see (1)). Spry was originally identified in Drosophila as a negative regulator of fibroblast growth factor (FGF) signaling during tracheal development (2). Subsequent studies have shown Spry to be a general inhibitor of growth factor-induced receptor tyrosine kinase (RTK) signaling pathways involved in Drosophila development and organogenesis (3-5). While Drosophila has only one *Spry* gene, at least four *Spry* homologues (*Spry*1-4) have been found in humans and mice (2;6-10). Mammalian Spry inhibit growth factor-induced cell responses, by inhibiting the RTK-dependent Ras/mitogen-activated protein (MAP) kinase signaling pathway (11-13). Several mechanisms for Spry inhibition of the RTK/Ras/MAP kinase pathway have been proposed, including blocking the interaction of the Grb2/SOS complex with the docking protein, FRS2 (3;11) or the inhibition of Raf (14-17). Another characteristic of the Spry inhibitors is their regulation by growth factors in a negative feedback loop, i.e. their expression is dependent on the signaling pathway that they regulate (18). Specifically, growth factors regulate both the level of Spry transcript (6) and in some systems, the recruitment of Spry proteins to the plasma membrane (19). However, unlike Spry1, Spry2 and Spry4 whose expression can be inhibited in response to downregulation of FGF signaling with the FGFR inhibitor SU5402, Spry3 is not inhibited by SU5402 treatment, suggesting that Spry3 might be involved in the regulation

of another signaling pathway (18;20). Indeed, recent reports indicate that Spry3 plays a role in axonal branching in a brain-derived neurotrophin factor dependent-manner (18;20); however there is very little information about the role of Spry3 in prostate cancer. Given that Spry 1, 2 and 4 proteins can inhibit FGF signaling, they can potentially decrease the biological activities of FGFs in prostate cancer cells and inhibit their ability to promote cancer progression.

We have previously shown by immunohistochemical and quantitative real-time PCR analysis that Spry1 and Spry4 are down-regulated in a subset of prostate cancers tissues when compared with normal prostate tissues (21;22). We have also demonstrated that transient and sustained increased expression of Spry1 markedly inhibits prostate cancer cell proliferation (21) whereas the sustained increased expression of Spry4 inhibited prostate cancer cell migration (22). McKie et al., (23) have observed that Spry2 expression is reduced in clinical prostate cancer tissues when compared with benign prostatic hyperplasia (BPH). The accumulating data indicates distinct differences in the functional roles for Spry1 and Spry4 in prostate cancer cell lines.

The decrease in Spry expression in the human prostate cancer, despite elevated levels of FGF ligands and FGF receptors, implies a loss of an important growth regulatory mechanism in prostate cancers that may potentiate the effects of increased FGF and FGFR expression in prostate cancer tissues and may represent a novel mechanism that facilitates aberrant RTK signaling in prostate carcinogenesis.

We have previously demonstrated that DNA methylation in the Spry1 promoter region is responsible for down-regulating Spry1 expression in prostate cancer (24). However, promoter methylation does not seem to explain gene silencing in all prostate cancer cases

studied. For instance, in some prostate cancer cases, where we detected low DNA methylation in the cancer tissues compared to the normal tissues, we also observed low Spry1 expression to suggest that other mechanisms of gene inactivation such as alterations in trans-acting factors and/or post-transcriptional activity may be responsible for the decreased expression in those cases.

The purpose of the present study is to investigate the relative contribution of trans-acting factors and post-transcriptional regulation of *Spry*1 in prostate cancer. Our data support a potential role of mir-21 in post-transcriptional regulation of *Spry*1 in prostate cancer cells.

MATERIALS AND METHODS

Cell Culture and treatment

The human prostate cancer cell lines, PC3, DU145 and LNCaP, and the immortalized normal prostate epithelial cell line pNT1A were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) unless otherwise stated.

LNCaP cells were treated with 10ng/ml of lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) for 24 hr in complete medium without antibiotics. Treated and untreated cells were harvested and extracted RNA used in quantitative RT-PCR with Cancer miRNA Array (SA BioSciences, Frederick, MD) and according to manufacturer's protocol.

Transfections and antibodies

LnCaP, PNT1A, DU145, and PC3 cells were transiently transfected with *Spry*1 construct (pcDNA-*Spry*1 (21)) or vector only (pcDNA3.1), shRNA-GATA-2, shRNA -GATA-4 , shRNA -EGR-1, shRNA -EGR-2 or vector only (PLKO), GATA 4 (pCMV6-GATA4; Origene) of EGR2 (pCMV6-EGR2; Origene) of the vector only (pCMV6; Origene). Cells were also transfected with anti-miR-21, or anti-miRNA negative control. All transfections were done using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and according to manufacturer's protocol. After 24, 48, or 72 h, cells were harvested and either miRNA was extracted using miRNA isolation kit (Ambion Inc., Austin, TX) or total RNA was extracted using TRIzol Reagent (Invitrogen). In addition, total protein

were extracted and used in Western blot analysis as previously described (21). All shRNAs and anti-miRs were purchased from Origene (Rockville, MD) and Applied Biosystems (Foster City, CA) respectively unless otherwise stated.

Antibodies used for western blotting, electrophoretic mobility shift assays and chromatin immunoprecipitation were the following: rabbit anti-Spry1, mouse anti-actin, rabbit anti–Egr-1, goat anti-Egr-2, goat anti–rabbit GATA-2, rabbit anti-GATA-4, rabbit anti-p-ERK ½ (Thr 202/Tyr 204), anti-PBX1 rabbit polyclonal IgG; anti-SP1 mouse monoclonal IgG; anti-HNF4a goat polyclonal IgG. Anti-acetyl-Histone H4 rabbit antiserum was purchased from Upstate Biotech (Lake Placid, NY). All antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA) unless otherwise stated.

cDNA Synthesis and Quantitative Real-Time PCR (RT-PCR)

Total RNA was used in first-strand DNA (cDNA) synthesis using Invitrogen SuperScript first-strand synthesis for reverse transcription-PCR and according to the manufacturer's protocol. Small species-enriched RNA (miRNA) was extracted and converted to cDNA using Taqman microRNA kit (Applied Biosystems) and according to manufacturer's instruction. Taqman primer sequences designed as corresponding to mir-21 were used and U6 was used as a normalizing control. Real-time PCR was carried out in a Bio-Rad iCycler real-time thermal cycler (Hercules, CA) as described previously (21) using LightCycler fast start DNA master cyber green I (Roche Applied Science). Expression of Spry1 and β-actin were evaluated according to manufacturer's protocol. Alternatively RT-PCR analysis of miRNA was carried out using the cDNA product along with

TaqMan primers and other PCR reagents in the PCR Universal Master Mix Kit (Applied Biosystems, Foster City, USA). The PCR conditions included initial incubation at 50^{o}C for 2 min and denaturing at 95^{o}C for 5 s and 60^{o}C for 1 min. Each sample was measured in duplicate.

Nuclear extracts and oligonucleotide probe preparation and Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared from PC3, DU145, LNCaP and PNT1A using RIPA lysis buffer (Thermo Scientific, Rockford IL). Oligonucleotide sequences were synthesized by Sigma Genosys. Oligonucleotide sequences encompassing binding sites for wild-type or mutant (mut) transcription factors were synthesized as sense (F) or antisense (R) strands: EGRF 5'-GGATCCAGCGGGGGCGAGCGGGGGCCA-3'; EGRR 5'-TGGCCCCCGCTCGCC-CCCGCTGGATCC-3'; mut- EGRF 5'-GGATCCATTTTTTTCGATTTTTTTCCA-3'; mutEGRR 5'-TGGAAAAAAATCGAAAAAAATGGATCC-3'; PBX1F 5'-CGAATTG-ATTGATGCACTAATTGGAG-3'; PBX1R 5'-CTCCAATTAGTGCATCAATCAATT-CG-3'; HNF4F 5'-ACAGGGTCAAAGGTCACGA-3'; HNF4R 5'-TCGTGACCTTGA-CCCTGT-3'; SP1F 5'-ATTCGATCGATCGGGGCGGGGCGAG-3'; SP1R 5'-CTCG-CCCCGCCCCGATCGAAT-3'. Each oligonucleotide sense strand was end-labeled with [γ-^{32}P] dATP (Amersham Biochemicals; 3000 Ci/mmol) and 1X polynucleotide kinase reaction buffer (Invitrogen). The labeled oligonucleotide was annealed to 50 fold molar excess of the complementary anti-sense strand by heating at 85^{o}C for 5 min and slowly cooling to room temperature. Unincorporated [γ-^{32}P] dATP were removed by purifying

the probes using a G-25 (Fine) Sephadex Quick spin columns (Roche). The EMSAs were carried out as described previously (25). Briefly, the ^{32}P-labeled oligonucleotide probes were incubated with or without nuclear extract in a total reaction volume of 25 µl containing the binding assay buffer (50 mM Tris-HCl, pH 7.4; 50 mM NaCl; 1 mg/ml BSA; 5 µg/ml poly dI-dC; 20% glycerol). The reactions were started by the addition of nuclear extract (5 µl per reaction) and incubated at room temperature for 30 minutes. Competition reactions were pre-incubated in the binding buffer for 30 min at room temperature with 100 fold molar excess of the corresponding unlabelled oligonucleotides followed by 30 min incubation at room temperature with the labeled oligonucleotide. For supershift analysis 1 µl of antibody was preincubated with the binding buffer for 45 minutes at room temperature prior to the addition of the ^{32}P-labeled probe. The bound and free DNA were resolved by electrophoresis through a 5% polyacrylamide gel at 175 V in 0.5 X TBE at room temperature for 2.5 h. Dried gels were exposed to Kodak Bio-Max film at -80^{o}C with intensifying screens.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously (26). For PCR reaction, DNA solution (50 ng) was used as a template with *Spry*1 Fwd 5 (forward) and Rev (reverse) primers as previously described (24).

RESULTS

Comparative sequence analysis of the Spry1 promoter locus

To characterize the *Spry*1 promoter region, we searched for transcription factor (TF) binding sites using the MatInspector program (27). We analyzed 2 kb of the genomic sequence upstream of the *Spry*1 transcription start site (24), using computer-based analysis (MatInspector software from Genomatix; www.Genomatix.de). We found potential binding sites for several TFs including GATA1 (28), EGR (29), SPI (30), PBX1 (31), and HNF4 (32) in the proximal promoter region. The human and murine *Spry*1 5'-flanking region upstream of their transcription start sites were aligned for sequence comparison. Over the entire 5'-flanking region of the human *Spry*1 promoter, only a very short region in the promoter (between -112 and +1 relative to the transcription) showed approximately 94% degree of homology with the mouse *Spry*1 promoter. As illustrated in Fig 1, Wilm's tumor (WT1) transcription factor binding sites: EGR1 and 3 (33), and WTE (34) are conserved between the two species. Interestingly, the nucleotide sequences immediately upstream from the EGR motif diverge in these species. Furthermore, we did not see any sequence homology between the human *Spry*1 promoter region and that of the published *Spry*2 (35) or *Spry*4 (36;37) promoters. The high sequence homology in the *Spry*1 promoter of the mouse and human indicates an evolutionary conserved mechanism(s) involving WT1 and EGR transcription factors in *Spry*1 gene regulation.

Electrophoretic mobility shift assay

To verify the binding interaction of the *Spry*1 consensus sequence *in vivo*, we performed electrophoretic mobility shift assay (EMSA) using designed consensus radiolabelled oligonucleotide probes to recognize EGR1, PBX1, HNF-4 and SP1 and nuclear extracts prepared from either LNCaP, PC-3 or pNT1A. Since all three cell lines demonstrated a similar band-shift pattern with each probe, only results using nuclear extracts from LNCaP cells are shown in Fig 2A. Three protein-DNA complexes (C1, C2 and C3) were formed with each of the oligonucleotide probes. These complexes represented sequence-specific interactions of proteins within this region, since the addition of 100-fold molar excess of the corresponding unlabelled oligonucleotide probe was able to compete away these complexes. To characterize these complexes further, supershift EMSA was conducted using specific antibodies. The addition of anti-SP1 antibody clearly abrogated the formation of C2, whereas the addition of anti-PBX1 and anti-HNF4 antibodies reduced the signal intensity of the respective C2 complex suggesting that the C2 complex is formed with SP1, PBX1 and HNF4 respectively. We did not see any significant effect of the addition of anti-EGR antibody on the protein-DNA complexes. However, when the EGR1 consensus binding sequence was mutated (Mut EGR1), we observed a new complex migrating very close with complex C2. Cold competition assay with wild-type EGR1 oligonucleotide competed out complex C2 totally but only partially competed the new complex. Furthermore, supershift assay successfully competed C2. This indicates that EGR1 protein preferentially recognize and interact with the wild-type EGR1 consensus binding sequence.

Chromatin immunoprecipitation (ChIP)

We next studied whether these TFs bound to the *Spry*1 promoter *in vivo* using ChIP assay. Figure 2B showed that indeed these TFs bound to *Spry*1 promoter *in vivo* as demonstrated by the same PCR product in the assay precipitation with different antibodies compared to the Anti-acetyl-Histone H4 antibody control (positive control). Conversely precipitation with normal goat IgG (negative control) did not show any binding. These studies clearly demonstrate that *Spry*1 proximal promoter region contain several sequence motifs (i.e., EGR, GATA, SP1, PBX1 and HNF4) which are specifically recognized by known as well as uncharacterized transcription factors and are functionally important and likely to be responsible for driving the basal transcription of the *Spry*1 gene.

Impact of EGR and GATA activity on Spry1 expression

To verify the involvement of transcription factor binding activity in regulating Spry1 expression, we investigated 2 transcription factors; EGR and GATA. We studied EGR because the EGR binding site appears to be highly conserved between human and mouse, and GATA because of its consensus binding site in Spry1 proximal promoter region. We carried out transient transfections in prostate cell lines with shRNA duplexes corresponding to EGR1, EGR2, GATA2 and GATA4. Western blot analysis were performed using Spry1 antibody and total cell lysates in order to examine the silencing effect of the EGR1, EGR2, GATA2 and GATA4 shRNA transfections on Spry1 protein expression. Since all shRNA transfections demonstrated a similar knockdown effect in the prostate cancer cells, only results from EGR2 and GATA4 shRNA transfections are shown (Fig 3a). On the other hand, when we over-expressed EGR2 and GATA4 in

prostate cell lines, this was accompanied by increase in Spry1 protein level as detected by Western blot analysis (Fig 3b). The results clearly showed that EGR2 and GATA4 expression co-relate with Spry1 expression as the blockade of EGR2 and GATA4 by small inhibitory RNA also inhibits Spry1 protein expression whereas the increased expression EGR2 and GATA4 resulted in increased expression of Spry1.

The role of miR21 expression and Spry1

Next, we assessed whether Spry1 expression is inhibited by microRNA because Spry1 mRNA contains several predicted micro-RNA binding sites (38). We focused on mir-21 because when LNCaP cells which expressed very low amounts of mir-21 (Fig 4a), were treated with bacterial lipopolysaccharide to induce inflammation, mir-21 was one miRNA that was significantly up-regulated (Fig 4b). When prostate cell lines were transiently transfected with anti-miR-21, we observed a significant increase in Spry1 protein expression when compared to scrambled control transfection (Fig. 4c). Because miRNAs have been shown to regulate mRNAs by binding to their 3'-UTRs and enhancing degradation or preventing translation (39), we reason that miR-21 affects Spry1 expression by degrading mRNA transcript and thereby repressing translation. Spry1 expression is dependent on the ERK signaling pathway (40), we therefore used phosphor-ERK1/2 as a marker for monitoring changes in Spry1 function that would be regulated by changes in its levels. As shown in figure 4c, increased expression of Spry1 in prostate cells in response to anti-miR-21 treatment led to a marked decrease in basal phosphor-ERK1/2 normalized to the total ERK. In other words, the increased expression of miR-21 in prostate cancer cells induces the down-regulation of

important inhibitory signals including Spry1 and this may potentially facilitate tumor progression.

DISCUSSION

In the present study, we have identified a highly conserved nucleotide binding site for the early growth response (EGR1) in the human and mouse *Spry*1 promoter region which underscore the importance of this motif in the regulation of the *Spry*1 gene expression. We have used EMSA and ChIP assays to demonstrate interaction of several cis-elements within the human *Spry*1 promoter region (including GATA2 & 4, EGR1 & 2, SP1, PBX1 and HNF4) that confers responsiveness to growth factor signaling. In the human prostate, there is strong evidence to suggest that EGR1 overexpression is involved in prostate cancer progression (41). For example, EGR1 expression levels are elevated in human prostate carcinomas in proportion to grade and stage. Whereas antisense oligonucleotides that block EGR1 function revert transformation of prostate cancer cells *in vitro* and delay prostate cancer progression *in vivo* (42). We have observed a direct correlation between Spry1 and EGR2 expression; a decrease in Spry1 expression in response to EGR2 knockdown whereas Spry1expression increased in response to EGR2 overexpression. Similarly, we observed a direct correlation of Spry1 expression with GATA4; knockdown of GATA4 decrease Spry1 expression, whereas the overexpression of GATA4 increased Spry1 expression in prostate cancer cell lines. One study has reported that GATA2 is expressed in substantial proportion of prostate cancers and that high level of GATA2 expression is associated with biochemical recurrence and distant metastatic progression (43). It appears that both EGR and GATA transcriptions are up-

regulated in prostate cancer even though Spry1 expression is down-regulated in a significant number of prostate cancer samples. Indeed several transcription factors with potential binding sites in the proximal regions of *Spry*1 promoter act as transcriptional activator (30-32) in prostate cancer. Thus, additional studies are needed in order to clarify the role of these transcription factors in *Spry*1 regulation in prostate cancer. However, the observation that *Spry*1 is a direct target for miR-21 regulation in addition to DNA methylation control (24) clearly demonstrate a strong epigenetic control for Spry1 expression in prostate cancer. One of the emerging fields in cancer research is miRNAs. Several miRNA expression studies and functional experiments in cancer have shown an important role for miRNAs in the disease initiation and progression and their potential as diagnostic, prognostic, and predictive biomarkers (37). In prostate cancer, higher levels of miR-21 have been reported in blood plasma of patients in comparison to healthy controls (38). This finding is in line with published data describing an oncogenic role of miR-21 in various cancers (39). One of the factors that could lead to increase expression of miR-21 in prostate cancer might be in response to chronic inflammation. The increased mir-21 expression in turn may exacerbate disease progression by repressing the translation of important regulatory genes including Spry1as described in this manuscript.

CONCLUSION

In the current studies, we have investigated the transcriptional and post-transcriptional regulation of *Spry*1 in human prostate cells. While *Spry*1 shows a highly conserved binding site for EGR and GATA, the role of these transcription factors in regulating *Spry*1 expression is not clear. On the other hand, we observe down-regulation

of Spry1 by miR-21 expression. Taken together with our previous observation that DNA methylation plays a role in Spry1 expression, our observation strongly indicate epigenetic mechanism plays an important role in *Spry*1 regulation in human prostate cancer.

ACKNOWLEDGEMENTS

This work was supported by grant from the Department of Defense Prostate Cancer Research Program New Investigator Award; PC040326 to Bernard Kwabi-Addo. The Department of Veterans Affairs Merit Review program to Michael Ittmann and the National Cancer Institute to the Baylor prostate cancer SPORE program (P50CA058204).

Reference List

(1) Kwabi-Addo B, Ozen M, Ittmann M. The role of fibroblast growth factors and their receptors in prostate cancer. Endocr Relat Cancer 2004 Dec;11(4):709-24.

(2) Hacohen N, Kramer S, Sutherland D, Hiromi Y, Krasnow MA. sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. Cell 1998 Jan 23;92(2):253-63.

(3) Casci T, Vinos J, Freeman M. Sprouty, an intracellular inhibitor of Ras signaling. Cell 1999 Mar 5;96(5):655-65.

(4) Kramer S, Okabe M, Hacohen N, Krasnow MA, Hiromi Y. Sprouty: a common antagonist of FGF and EGF signaling pathways in Drosophila. Development 1999 Jun;126(11):2515-25.

(5) Reich A, Sapir A, Shilo B. Sprouty is a general inhibitor of receptor tyrosine kinase signaling. Development 1999 Sep;126(18):4139-47.

(6) Minowada G, Jarvis LA, Chi CL, Neubuser A, Sun X, Hacohen N, et al. Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. Development 1999 Oct;126(20):4465-75.

(7) de Maximy AA, Nakatake Y, Moncada S, Itoh N, Thiery JP, Bellusci S. Cloning and expression pattern of a mouse homologue of drosophila sprouty in the mouse embryo. Mech Dev 1999 Mar;81(1-2):213-6.

(8) de Maximy AA, Nakatake Y, Moncada S, Itoh N, Thiery JP, Bellusci S. Cloning and expression pattern of a mouse homologue of Drosophila sprouty in the mouse embryo. Mechanisms of Development 1999 Mar;81(1-2):213-6.

(9) Hacohen N, Kramer S, Sutherland D, Hiromi Y, Krasnow MA. sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. Cell 1998 Jan 23;92(2):253-63.

(10) Minowada G, Jarvis LA, Chi CL, Neubuser A, Sun X, Hacohen N, et al. Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. Development 1999 Oct;126(20):4465-75.

(11) Hanafusa H, Torii S, Yasunaga T, Nishida E. Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. Nat Cell Biol 2002 Nov;4(11):850-8.

(12) Gross I, Bassit B, Benezra M, Licht JD. Mammalian sprouty proteins inhibit cell growth and differentiation by preventing ras activation. J Biol Chem 2001 Dec 7;276(49):46460-8.

(13) Yigzaw Y, Cartin L, Pierre S, Scholich K, Patel TB. The C terminus of sprouty is important for modulation of cellular migration and proliferation. J Biol Chem 2001 Jun 22;276(25):22742-7.

(14) Impagnatiello MA, Weitzer S, Gannon G, Compagni A, Cotten M, Christofori G. Mammalian sprouty-1 and -2 are membrane-anchored phosphoprotein inhibitors of growth factor signaling in endothelial cells. J Cell Biol 2001 Mar 5;152(5):1087-98.

(15) Sasaki A, Taketomi T, Wakioka T, Kato R, Yoshimura A. Identification of a dominant negative mutant of Sprouty that potentiates fibroblast growth factor- but not epidermal growth factor-induced ERK activation. J Biol Chem 2001 Sep 28;276(39):36804-8.

(16) Impagnatiello MA, Weitzer S, Gannon G, Compagni A, Cotten M, Christofori G. Mammalian sprouty-1 and -2 are membrane-anchored phosphoprotein inhibitors of growth factor signaling in endothelial cells. J Cell Biol 2001 Mar 5;152(5):1087-98.

(17) Sasaki A, Taketomi T, Kato R, Saeki K, Nonami A, Sasaki M, et al. Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. Nat Cell Biol 2003 May;5(5):427-32.

(18) Kim HJ, Bar-Sagi D. Modulation of signalling by Sprouty: a developing story. Nat Rev Mol Cell Biol 2004 Jun;5(6):441-50.

(19) Lim J, Yusoff P, Wong ES, Chandramouli S, Lao DH, Fong CW, et al. The cysteine-rich sprouty translocation domain targets mitogen-activated protein kinase inhibitory proteins to phosphatidylinositol 4,5-bisphosphate in plasma membranes. Mol Cell Biol 2002 Nov;22(22):7953-66.

(20) Panagiotaki N, Dajas-Bailador F, Amaya E, Papalopulu N, Dorey K. Characterisation of a new regulator of BDNF signalling, Sprouty3, involved in axonal morphogenesis in vivo. Development 2010 Dec;137(23):4005-15.

(21) Kwabi-Addo B, Wang J, Erdem H, Vaid A, Castro P, Ayala G, et al. The expression of Sprouty1, an inhibitor of fibroblast growth factor signal transduction, is decreased in human prostate cancer. Cancer Res 2004 Jul 15;64(14):4728-35.

(22) Wang J, Thompson B, Ren C, Ittmann M, Kwabi-Addo B. Sprouty4, a suppressor of tumor cell motility, is down regulated by DNA methylation in human prostate cancer. Prostate 2006 May 1;66(6):613-24.

(23) McKie AB, Douglas DA, Olijslagers S, Graham J, Omar MM, Heer R, et al. Epigenetic inactivation of the human sprouty2 (hSPRY2) homologue in prostate cancer. Oncogene 2005 Mar 24;24(13):2166-74.

(24) Kwabi-Addo B, Ren C, Ittmann M. DNA methylation and aberrant expression of Sprouty1 in human prostate cancer. Epigenetics 2009 Jan;4(1):54-61.

(25) Zimarino V, Wu C. Induction of sequence-specific binding of Drosophila heat shock activator protein without protein synthesis. Nature 1987 Jun 25;327(6124):727-30.

(26) Rosa AL, Wu YQ, Kwabi-Addo B, Coveler KJ, Reid S, V, Shaffer LG. Allele-specific methylation of a functional CTCF binding site upstream of MEG3 in the human imprinted domain of 14q32. Chromosome Res 2005;13(8):809-18.

(27) Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res 1995 Dec 11;23(23):4878-84.

(28) Gong QH, Stern J, Dean A. Transcriptional role of a conserved GATA-1 site in the human epsilon-globin gene promoter. Mol Cell Biol 1991 May;11(5):2558-66.

(29) Eid MA, Kumar MV, Iczkowski KA, Bostwick DG, Tindall DJ. Expression of early growth response genes in human prostate cancer. Cancer Res 1998 Jun 1;58(11):2461-8.

(30) Ibanez-Tallon I, Ferrai C, Longobardi E, Facetti I, Blasi F, Crippa MP. Binding of Sp1 to the proximal promoter links constitutive expression of the human uPA gene and invasive potential of PC3 cells. Blood 2002 Nov 1;100(9):3325-32.

(31) Kamps MP, Murre C, Sun XH, Baltimore D. A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. Cell 1990 Feb 23;60(4):547-55.

(32) Svoboda M, Riha J, Wlcek K, Jaeger W, Thalhammer T. Organic anion transporting polypeptides (OATPs): regulation of expression and function. Curr Drug Metab 2011;12(2):139-53.

(33) Morris JF, Madden SL, Tournay OE, Cook DM, Sukhatme VP, Rauscher FJ, III. Characterization of the zinc finger protein encoded by the WT1 Wilms' tumor locus. Oncogene 1991 Dec;6(12):2339-48.

(34) Nakagama H, Heinrich G, Pelletier J, Housman DE. Sequence and structural requirements for high-affinity DNA binding by the WT1 gene product. Mol Cell Biol 1995 Mar;15(3):1489-98.

(35) Ding W, Bellusci S, Shi W, Warburton D. Functional analysis of the human Sprouty2 gene promoter. Gene 2003 Dec 11;322:175-85.

(36) Ding W, Bellusci S, Shi W, Warburton D. Genomic structure and promoter characterization of the human Sprouty4 gene, a novel regulator of lung morphogenesis. Am J Physiol Lung Cell Mol Physiol 2004 Jul;287(1):L52-L59.

(37) Ding W, Bellusci S, Shi W, Warburton D. Genomic structure and promoter characterization of the human Sprouty4 gene, a novel regulator of lung morphogenesis. Am J Physiol Lung Cell Mol Physiol 2004 Jul;287(1):L52-L59.

(38) Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, et al. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature 2008 Dec 18;456(7224):980-4.

(39) Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009 Jan 23;136(2):215-33.

(40) Ozaki K, Kadomoto R, Asato K, Tanimura S, Itoh N, Kohno M. ERK pathway positively regulates the expression of Sprouty genes. Biochem Biophys Res Commun 2001 Aug 3;285(5):1084-8.

(41) Baron V, Duss S, Rhim J, Mercola D. Antisense to the early growth response-1 gene (Egr-1) inhibits prostate tumor development in TRAMP mice. Ann N Y Acad Sci 2003 Dec;1002:197-216.

(42) Baron V, De GG, Krones-Herzig A, Virolle T, Calogero A, Urcis R, et al. Inhibition of Egr-1 expression reverses transformation of prostate cancer cells in vitro and in vivo. Oncogene 2003 Jul 3;22(27):4194-204.

(43) Bohm M, Locke WJ, Sutherland RL, Kench JG, Henshall SM. A role for GATA-2 in transition to an aggressive phenotype in prostate cancer through modulation of key androgen-regulated genes. Oncogene 2009 Oct 29;28(43):3847-56.

FIGURE LEGENDS

Fig. 1. Alignment of sequence in the 5'-flanking region of human and murine *Spry*1 gene. The nucleotide sequences surrounding the transcription start site and the 5'-flanking region were compared for human and mouse *Spry*1 gene. The putative binding sites for indicated transcription factors, which are conserved in both species, are boxed. An asterics (*) indicate core similarity of 1.000 with human sequence.

Fig. 2. Identification of transcription factors binding to the *Spry*1 promoter. A. The analysis includes EGR1, SP1, PBX1 and HNF4. Radiolabelled double-stranded DNA

oligonucleotides (probes) were incubated with or without nuclear extracts from LNCaP cells. Protein-DNA complex is indicated (C1, C2, C3), free or unbound probe is indicated at the bottom. Specificity of DNA-protein complex was investigated using 100 fold molar excess of corresponding unlabelled probe shown as competitor or the corresponding antibody shown as supershift. **B**. Chromatin immunoprecipitation assays shows in vivo binding of different antibodies to the proximal *Spry*1 promoter. Anti-acetyl-Histone H4 antibody binding to DNA is used as a positive control (lane 3). Lanes 2 and 4 shows no amplification in the water and the normal IgG negative controls respectively.

Fig. 3. The effect of EGR2 and GATA4 expression on Spry1 protein level. A. shRNA knock-down of EGR2 and GATA4 and Spry1 expression in prostate cell lines. Prostate cell lines were transiently transfected with 250 pmol of shEGR2 (+) or vector only (-) transfection (top panel). In the lower panel prostate cell lines were transiently transfected with GATA4 (+) or vector only (-) transfection. After the transfection for 48 h, protein extracts were collected from cells and subjected to Western blotting with anti-Spry1 antibody, anti-EGR2 antibody, anti-GATA4 antibody or control β-actin antibody. Each transfection was done in duplicate. **B.** Over-expression of EGR2 and GATA4 and Spry1 expression in prostate cell lines. Prostate cell lines were transiently transfected with EGR2 expression vector (+) or vector only (-; top panel). In the lower panel prostate cell lines were transiently transfected with GATA4 expression (+) or vector only (-). After the transfection for 48 h, protein extracts were collected from cells and subjected to Western blotting with anti-Spry1 antibody, anti-EGR2 antibody, anti-GATA4 antibody or control β-actin antibody. Each transfection was done in duplicate.

Fig. 4. The effect of miR-21 on Spry1 and pERK1/2 expression. A. Comparison of miR-21 levels in DU145, PC3, LNCaP, PNT1A and RWPE1 cells. miRNAs was extracted from each cell line and subjected to RT-PCR analysis using Taqman primer sequences designed to correspond to miR-21 as described in materials and methods. **B.** Expression levels of miRNAs after LNCaP cells were treated with 10ng/ml of LPS for 24 h. The miRNA Array expression was examined using quantitative RT-PCR as described in materials and methods. **C.** Prostate cell lines were transiently transfected with anti-miR-21 (+) or anti-miR negative control (-) at 250 pmol. After the transfection for 48 h, protein extracts were collected from cells and subjected to Western blotting with anti-Spry1 antibody, anti-pERK1/2 antibody or control β-actin antibody. Each transfection was done in duplicate.

Figure 1

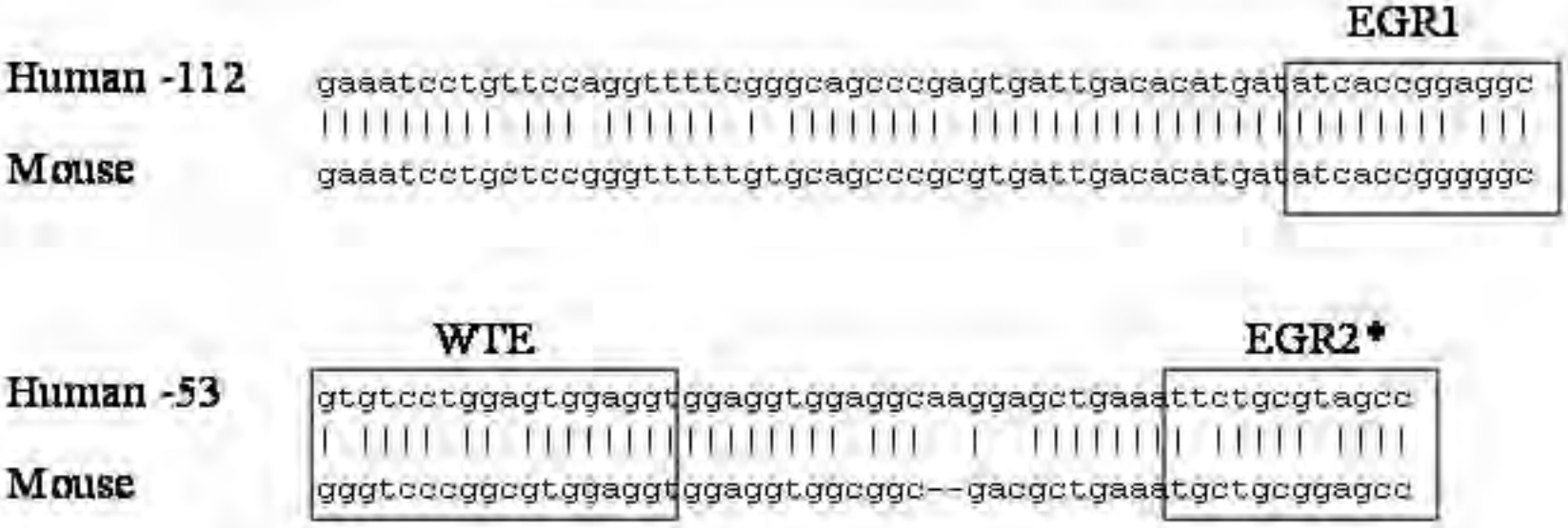

EGR1
Human -112
gaaatcctgttccaggttttcgggcagcccgagtgattgacacatgatatcaccggaggc
Mouse
gaaatcctgctccgggtttttgtgcagcccgcgtgattgacacatgatatcaccgggggc
WTE
EGR2*
Human -53
gtgtcctggagtggaggtggaggtggaggcaaggagctgaaattctgcgtagcc
Mouse
gggtcccggcgtggaggtggaggtggcggc--gacgctgaaatgctgcggagcc

Figure 2A

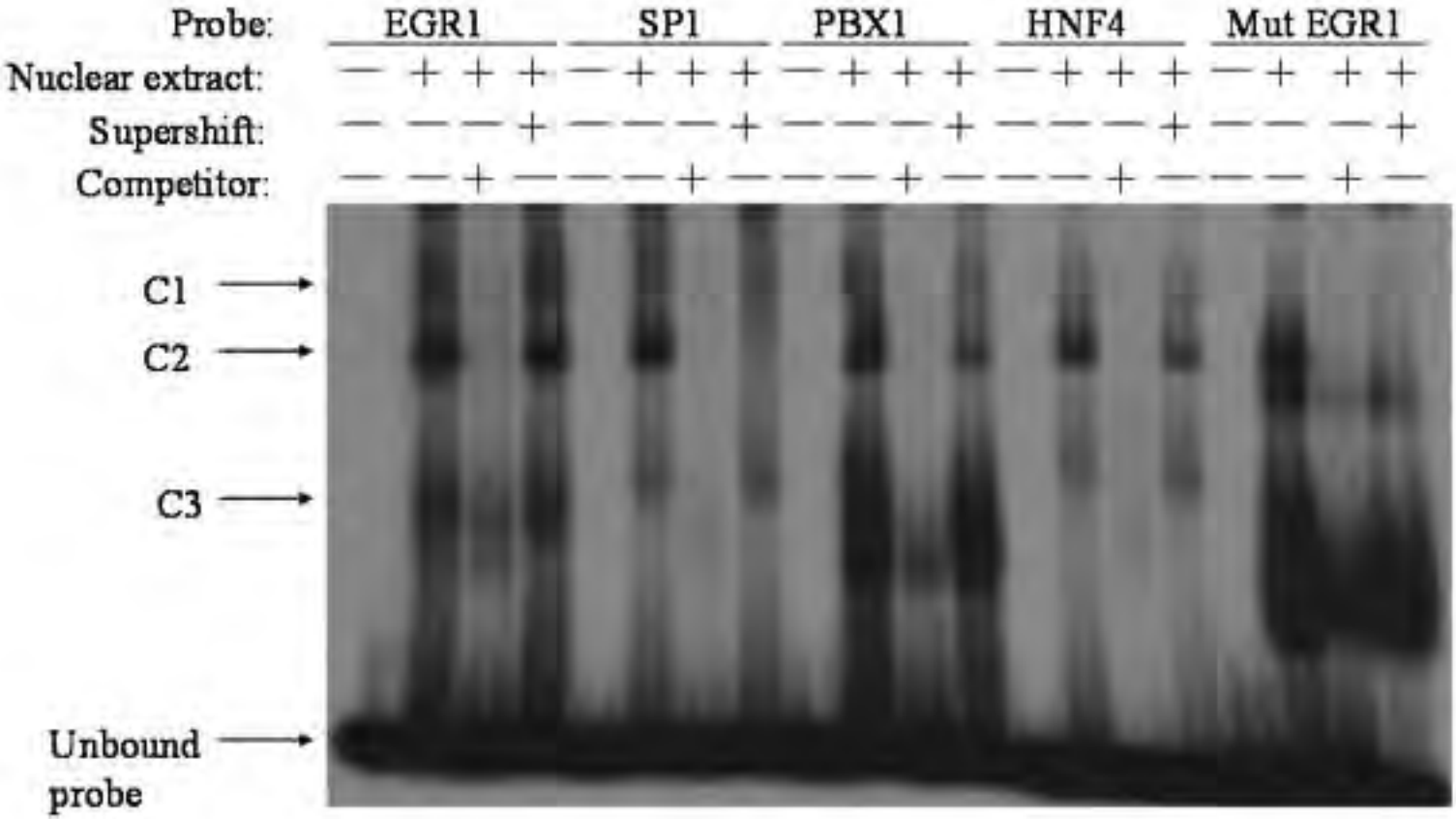

Probe:
EGR1
SP1
PBX1
HNF4
Mut EGR1
Nuclear extract:
Supershift:
Competitor:
C1
C2
C3
Unbound probe

Figure 2B

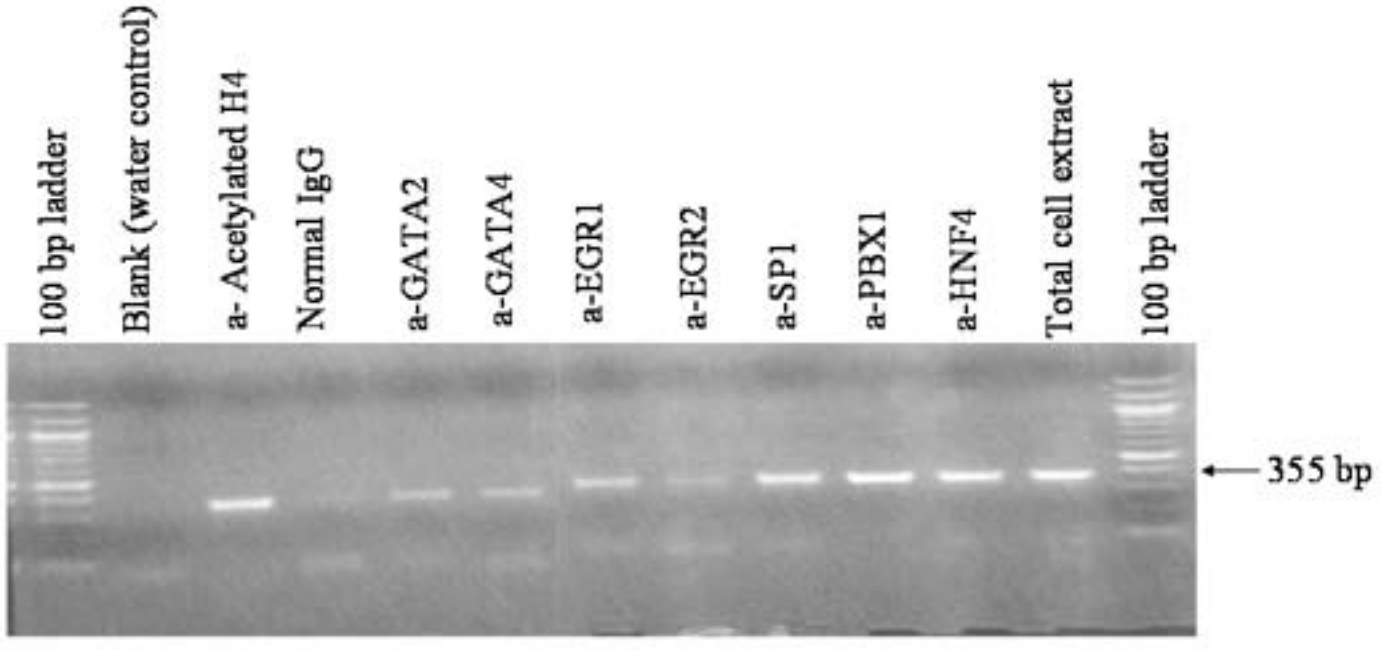

100 bp ladder
Blank (water control)
a- Acetylated H4
Normal IgG
a-GATA2
a-GATA4
a-EGR1
a-EGR2
a-SP1
a-PBX1
a-HNF4
Total cell extract
100 bp ladder
355 bp

Figure 3A

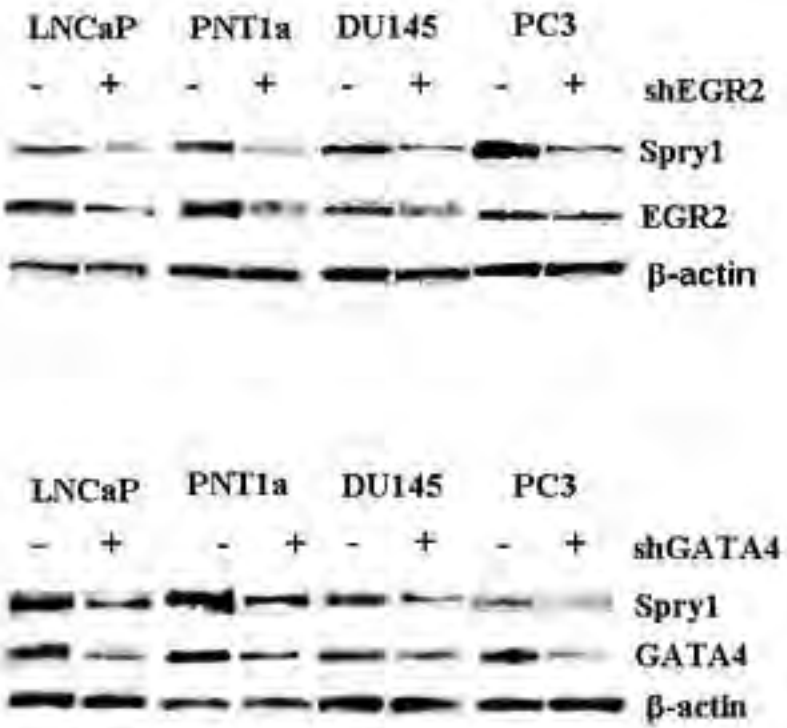

LNCaP
PNT1a
DU145
PC3
- + - + - + - +
shEGR2
Spry1
EGR2
β-actin
LNCaP
PNT1a
DU145
PC3
- + - + - + - +
shGATA4
Spry1
GATA4
β-actin

Figure 3B

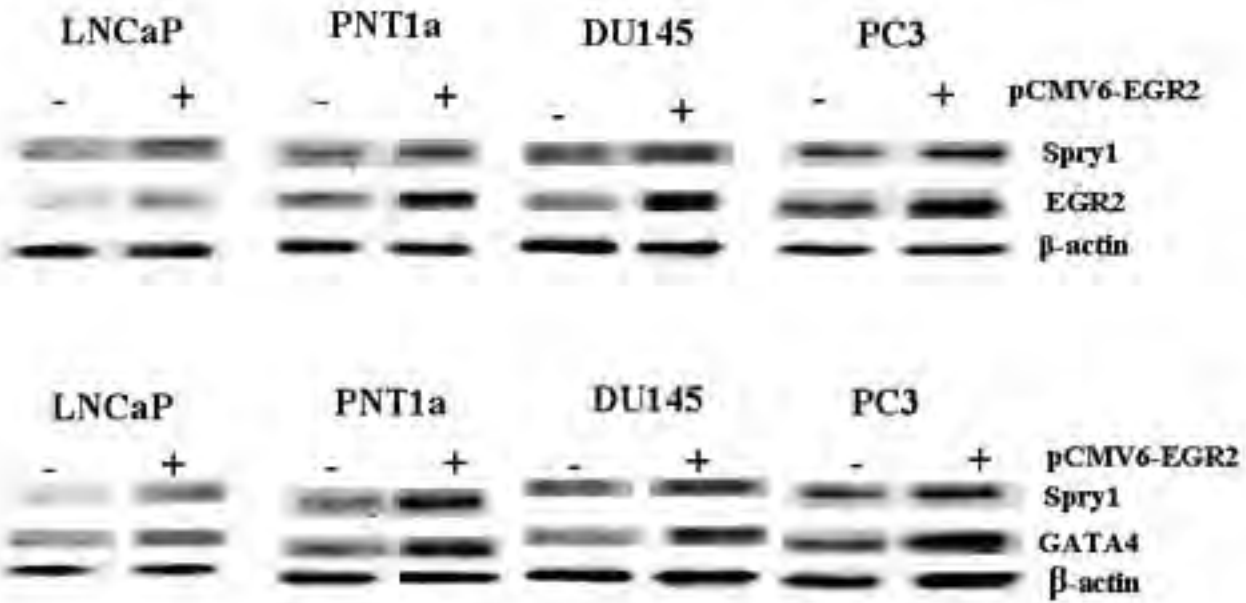

LNCaP
PNT1a
DU145
PC3
- + - + - + - +
pCMV6-EGR2
Spry1
EGR2
β-actin
LNCaP
PNT1a
DU145
PC3
- + - + - + - +
pCMV6-EGR2
Spry1
GATA4
β-actin

Figure 4A

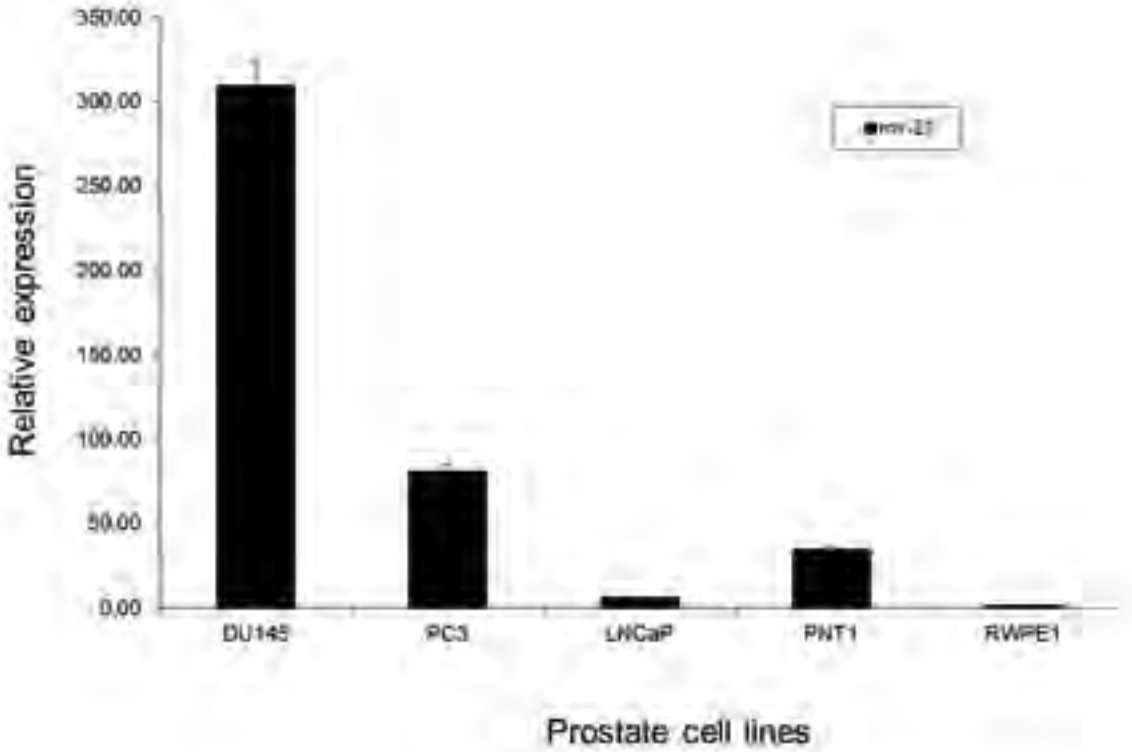

350,00
300,00
250,00
200,00
150,00
100,00
50,00
0,00
Relative expression
DU145
PC3
LNCaP
PNT1
RWPE1
Prostate cell lines

Figure 4B

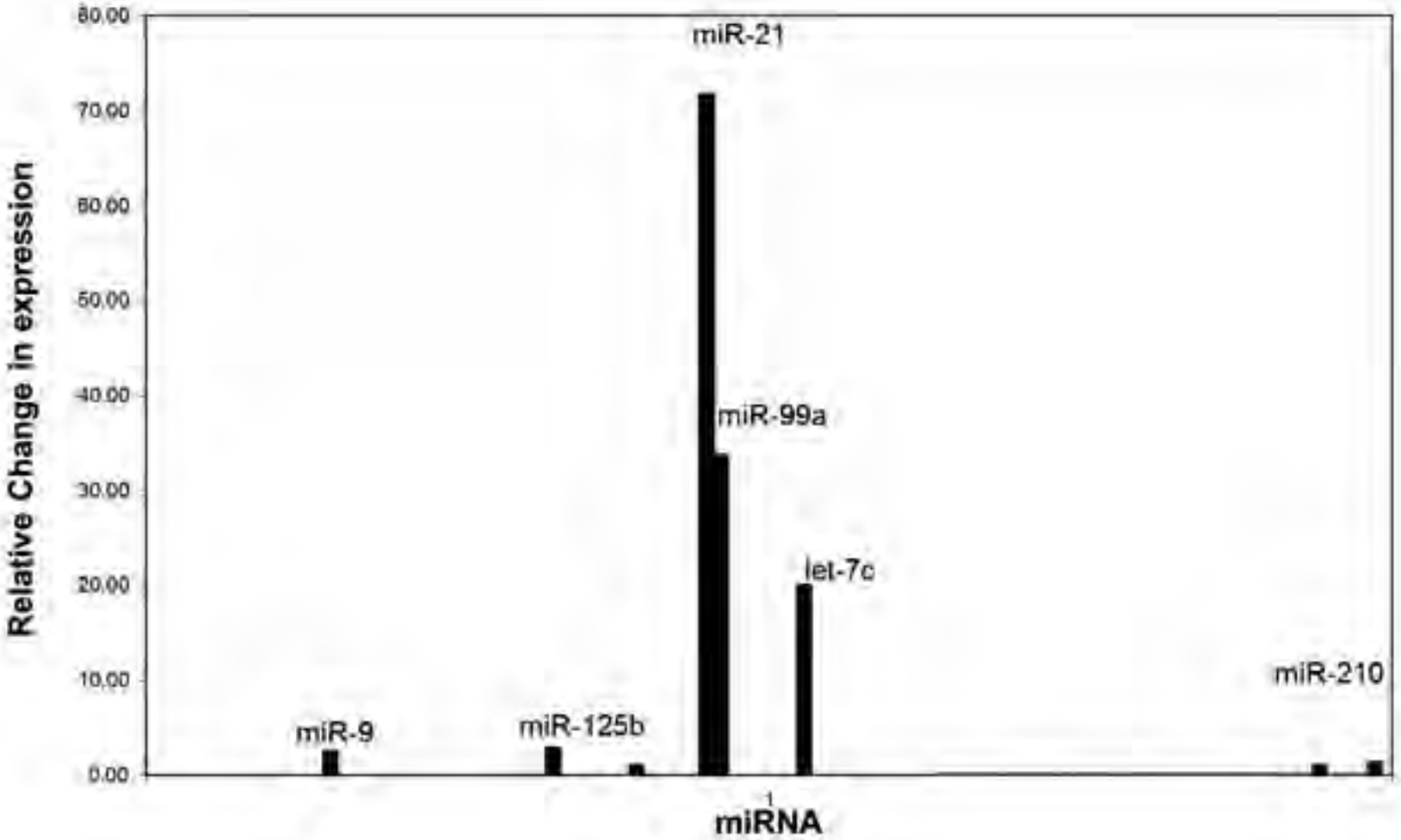
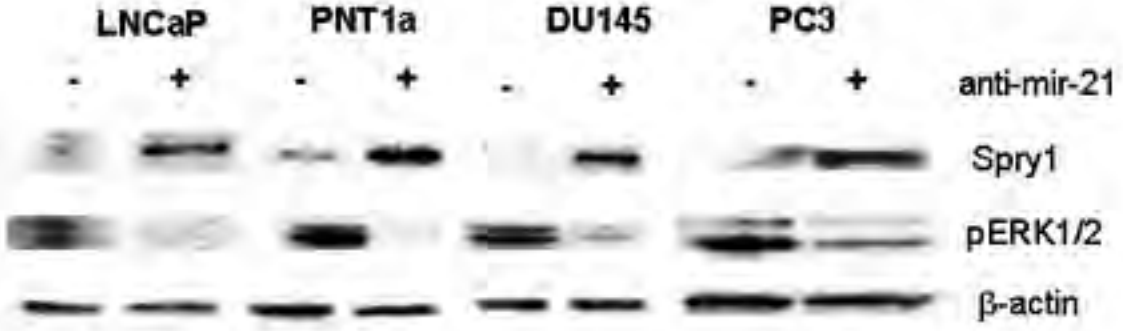

80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Relative Change in expression
miR-21
miR-99a
let-7c
miR-210
miR-9
miR-125b
miRNA

Figure 4C



Clinical Cancer Research

Identification of Differentially Methylated Genes in Normal Prostate Tissues from African American and Caucasian Men

Bernard Kwabi-Addo, Songping Wang, Woonbok Chung, et al.

Clin Cancer Res 2010;16:3539-3547. Published OnlineFirst July 6, 2010.

Published OnlineFirst July 6, 2010; DOI:10.1158/1078-0432.CCR-09-3342

Human Cancer Biology

Clinical Cancer Research

Identification of Differentially Methylated Genes in Normal Prostate Tissues from African American and Caucasian Men

Bernard Kwabi-Addo[1], Songping Wang[1], Woonbok Chung[5], Jaroslav Jelinek[5], Steven R. Patierno[3], Bi-Dar Wang[3], Ramez Andrawis[4], Norman H. Lee[3], Victor Apprey[2], Jean-Pierre Issa[5], and Michael Ittmann[6]

Abstract

Purpose: Aberrant DNA methylation changes are common somatic alterations in prostate carcinogenesis. We examined the methylation status of six genes in prostate tissue specimens from African American (AA) and Caucasian (Cau) males.

Experimental Design: We used pyrosequencing to quantitatively measure the methylation status of *GSTP1*, *AR*, *RARβ2*, *SPARC*, *TIMP3*, and *NKX2-5*. Real-time PCR was used to determine gene expression, and gene reactivation was analyzed by 5-aza-2′-deoxycytidine and/or trichostatin A treatment.

Results: Statistical analysis showed significantly higher methylation in the prostate cancer tissue samples in comparison with matched normal samples for *GSTP1* ($P = 0.0001$ for AA; $P = 0.0008$ for Cau), *RARβ2* ($P < 0.001$ for AA and Cau), *SPARC* ($P < 0.0001$ for AA and Cau), *TIMP3* ($P < 0.0001$ for AA and Cau), and *NKX2-5* ($P < 0.0001$ for AA; $P = 0.003$ for Cau). Overall, we observed significant differences ($P < 0.05$) in the methylation level for all genes, except *GSTP1*, in the AA samples in comparison with the Cau samples. Furthermore, regression analysis revealed significantly higher methylation for *NKX2-5* ($P = 0.008$) and *TIMP3* ($P = 0.039$) in normal prostate tissue samples from AA in comparison with Cau, and a statistically significant association of methylation with age for *NKX2-5* ($P = 0.03$) after adjusting for race.

Conclusion: Our findings show higher methylation of several genes in prostate tissue samples from AA in comparison with Cau and may potentially contribute to the racial differences that are observed in prostate cancer pathogenesis. *Clin Cancer Res; 16(14); 3539–47. ©2010 AACR.*

Prostate cancer (PCa) is a common malignancy and a leading cause of cancer death among men in the United States. PCa cells are known to carry a variety of genetic defects, including gene mutations, deletions, translocations, and amplifications, which endow the cells with new capabilities for dysregulated proliferation, inappropriate survival, tissue invasion and destruction, immune system evasion, and metastasis (1). More recently, it has become apparent that PCa cells also carry epigenetic defects, including changes in cytosine methylation patterns and chromatin structure and/organization, which are equivalent to genetic changes effecting and maintaining neoplastic and malignant phenotypes (2). For human PCa, abundant evidence has accumulated to suggest that somatic epigenetic alterations may appear earlier during cancer development, as well as more commonly and consistently, than genetic changes (3). Furthermore, epigenetic changes tend to arise in association with age (4) and/or in response to chronic or recurrent inflammation leading to cell and tissue damage (5).

Epigenetic inactivation of genes in cancer cells is largely based on transcriptional silencing by aberrant CpG methylation of CpG-rich promoter regions (6, 7). Aberrant promoter methylation of *GSTP1*, encoding the π-class glutathione *S*-transferase (GST), an enzyme capable of detoxifying electrophilic and oxidant carcinogens, remains the most common somatic genome abnormality (>90% of cases) reported thus far for PCa, appearing earlier and more frequently than other gene defects that arise during PCa development (8). Since the recognition that the *GSTP1* CpG was frequently hypermethylated in PCa, more than 40 genes have been reported to be targets of DNA hypermethylation–associated epigenetic gene silencing in PCa cells (9). Despite the increasing number of aberrantly methylated genes in PCa, only a few genes show promise as PCa biomarkers for early diagnosis and disease risk assessment.

In this study, we sought to investigate DNA methylation changes in prostate tissue samples from African American

Authors' Affiliations: [1]Department of Medicine, Cancer Center, and [2]Statistical Genetics and Bioinformatics Unit, Howard University College of Medicine; [3]Department of Pharmacology and Physiology and [4]Department of Urology, George Washington University Medical Center, Washington, District of Columbia; [5]Department of Leukemia, The University of Texas M.D. Anderson Cancer Center; and [6]Department of Pathology, Baylor College of Medicine, Houston, Texas

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Bernard Kwabi-Addo, Department of Medicine, Howard University Cancer Center, 2041 Georgia Avenue N.W., Washington, DC 20060. Phone: 202-806-9149; Fax: 202-667-1686; E-mail: bkwabi-addo@howard.edu.

doi: 10.1158/1078-0432.CCR-09-3342

Published OnlineFirst July 6, 2010; DOI:10.1158/1078-0432.CCR-09-3342

Translational Relevance

Prostate cancer (PCa) cells carry a myriad of genomic defects including genetic and epigenetic alterations. Epigenetic changes such as DNA methylation appear early and are more common and consistent than genetic defects. This makes DNA methylation changes very attractive as biomarkers for PCa detection. Furthermore, because epigenetic DNA methylation changes are reversible, epigenetic drugs are being explored for reversing somatic epigenetic defects. Identification of novel DNA methylation genes that serve as biomarkers would improve future clinical practice in PCa disease detection. In addition, the prevalence of DNA methylation changes in different ethnic groups could potentially lead to designing "ethnic-sensitive" biomarkers and epigenetic drugs for PCa. Our study describes the methylation status of regulatory genes in prostate tissues from African Americans and Caucasians and discusses how the prevalence of methylation changes can help us to understand PCa disparity and lead to the design of ethnic-sensitive biomarkers for PCa detection.

(AA) men in comparison with Caucasian (Cau) men to identify methylated genes that could be potentially useful as "ethnic-sensitive" biomarkers for the detection of PCa.

Materials and Methods

Cell lines and tissue samples

The human PCa cell lines PC3, DU145, and LNCaP were obtained from the American Type Culture Collection. The immortalized normal prostate epithelial cell line pNT1A was obtained from the European Collection of Cell Culture. All cell lines were cultured in the recommended medium in the presence of 10% fetal bovine serum (Invitrogen) unless otherwise stated. We collected matched pairs of normal and PCa tissue samples from both AA and Cau male patients with PCa (Table 1A; age range, 52-75 years) at the time of radical prostatectomy. The prostate tissue samples were obtained with informed consent and maintained by the Baylor Specialized Program of Research Excellence in the PCa tissue bank. The pathologic status was confirmed before processing, and we chose tumor samples that had >70% cancer, and in some cases we needed to microdissect to get that level of enrichment. Matched normal samples were confirmed free of tumor by histologic examination. In addition, normal prostate tissue samples were obtained from the George Washington University Hospital pathology tissue bank. The samples were collected by transrectal ultrasound needle biopsy from men with an elevated serum PSA level of ≥4 ng/mL and an abnormality detected by digital rectal examination. Needle biopsies from AA and Cau males (age range, 47-85 years) were determined to be noncancerous. The mean age was 62.7 ± 7.2 and 62.4 ± 8.8 years ($P = 0.89$) for AA and Cau, respectively. Fifteen of 18 and 12 of 13 biopsies ($P = 0.53$) were from the left mid-zone of the prostate of AA and Cau, respectively. The remaining samples were either from the left apex or the right mid-zone (Table 1B).

Bisulfite modification, PCR, and pyrosequencing analysis

High molecular weight genomic DNA extracted from prostate tissues and WBC was modified using sodium bisulfite treatment (10). Briefly, genomic DNA (2 μg) was denatured in 0.3 mol/L NaOH at 37°C for 15 minutes; sodium bisulfite and hydroquinone were added to final concentrations of 3.1 mol/L and 0.5 mmol/L, respectively. The reaction was incubated at 50°C for 16 hours and desalted using Wizard DNA purification resin (Promega) according to the instruction of the manufacturer. Bisulfite modification was completed by DNA desulfonation in 0.3 mol/L NaOH at 37°C for 15 minutes. Modified DNA was precipitated with ethanol, washed in 70% ethanol, dried, and dissolved in 50 μL of TE buffer. The PCR primers were designed to assay the methylation status of CpGs within 0.5 kb from the transcription start site (primer sequences are shown in Table 2). The CpG islands interrogated are shown in Supplementary Data. Either one-step or two-step PCR reactions were carried out using 2 μL of bisulfite-converted genomic DNA and either one or two sets of different bisulfite PCR primers in a standard PCR reaction mix. One of the primers (reverse primer) in the final PCR reaction was biotinylated to create a ssDNA template for the pyrosequencing reaction. Where indicated, we used a previously described amplification protocol (11) based on the universal primer approach. Briefly, the biotinylated reverse primer was substituted with a 5′ tailed unlabeled reverse primer and a biotinylated universal primer at a ratio of 1:9 in the PCR reaction. The integrity of the PCR product was verified on 1.5% agarose gels with ethidium bromide staining. The PCR product was immobilized on streptavidin-Sepharose beads (Amersham), washed, and denatured, and the biotinylated strands were released into annealing buffer containing the sequencing primer. Pyrosequencing was done using the PSQ HS96 Gold SNP Reagents on a PSQ 96HS machine (Qiagen). PCR primer sequences and sequencing primer sequences are listed in Table 2. Bisulfite-converted DNA from blood of normal volunteers and blank reactions, with water substituted for DNA, served as negative control and bisulfite-converted *Sss*I methylase–treated blood DNA served as a positive control. Each bisulfite PCR and pyrosequencing reaction was done at least twice.

RNA extraction, cDNA preparation, and reverse transcription-PCR

Total RNA extracted from cells and prostate tissues using TRIzoL reagent (Invitrogen) was used in cDNA synthesis using the Invitrogen SuperScript first-strand synthesis

Published OnlineFirst July 6, 2010; DOI:10.1158/1078-0432.CCR-09-3342

Table 1. Patient clinical and demographic characteristics by race

A

	AA	Cau
T2	17 (44)	28 (43)
T3a	18 (46)	31 (58)
T3b	4 (10)	6 (9)
N1	0 (0)	2 (3)
Gleason	6.4	6.8

B

No.	AA				Cau			
	Core	PCa GS	PSA (ng/mL)	Age (y)	Core	PCa GS	PSA (ng/mL)	Age (y)
1	RM	NEG (GS = 0)	0.5	54	LM	NEG (GS = 0)	1.5	65
2	LM	NEG (GS = 0)	0.2	63	LA	NEG (GS = 0)		58
3	LA	NEG (GS = 0)	6.3	60	LM	NEG (GS = 0)	4.1	65
4	LM	NEG (GS = 0)	4.9	65	LM	NEG (GS = 0)	6.2	59
5	LA	NEG (GS = 0)	3.6	68	LM	NEG (GS = 0)	3.8	60
6	LM	NEG (GS = 0)	1.5	71	LM	NEG (GS = 0)	4.1	59
7	LM	NEG (GS = 0)	5.7	61	LM	NEG (GS = 0)	5.1	76
8	LM	NEG (GS = 0)	4.8	47	LM	NEG (GS = 0)	4.9	64
9	LM	NEG (GS = 0)	0.5	67	LM	NEG (GS = 0)	8.7	85
10	LM	NEG (GS = 0)	6.2	65	LM	NEG (GS = 0)	9.0	56
11	LM	NEG (GS = 0)	1.4	65	LM	NEG (GS = 0)	4.9	72
12	LM	NEG (GS = 0)	4.9	70	LM	NEG (GS = 0)	2.1	59
13	LM	NEG (GS = 0)	4.7	59	LM	NEG (GS = 0)	1.7	55
14	LM	NEG (GS = 0)	7.7	54				
15	LM	NEG (GS = 0)	9.3	61				
16	LM	NEG (GS = 0)	14.1	55				
17	LM	NEG (GS = 0)	5.0	76				
18	LM	NEG (GS = 0)	5.0	68				

NOTE: A, matched pairs of normal and PCa tissue samples obtained from AA and Cau male patients with PCa at the time of radical prostatectomy. The staging (T) and Gleason score are shown. Data are number of cases with percentage in parentheses. N1 is positive pelvic lymph nodes (age range, 52-75 y). B, needle biopsies of normal prostate tissue samples from AA and Cau male patients (age range, 47-85 y) with elevated serum PSA level and an abnormality detected by digital rectal examination. The mean age was 62.7 ± 7.2 and 62.4 ± 8.8 y ($P = 0.89$) for the AA and Cau groups, respectively. Fifteen of 18 and 12 of 13 biopsies ($P = 0.53$) were from the left mid-zone of the prostate of AA and Cau samples, respectively. The remaining samples were either from the left apex or the right mid-zone. Gleason score of 0 indicates noncancerous.
Abbreviations: GS, Gleason score; LA, left apex; LM, left mid-zone; RM, right mid-zone; NEG, Negative.

system for reverse transcription-PCR (RT-PCR) and according to the manufacturer's protocol (primers used are listed in Table 2). Either SYBR Green or TaqMan assay (designed for *NKX2-5*) was used to quantitatively measure mRNA expression. Real-time PCR was carried out in a Bio-Rad iCycler real-time thermal cycler as described previously (12) and incorporating optimized PCR reaction conditions for each gene. First, a DNA standard was generated for each gene using PCR amplification of the coding sequence. The PCR product concentration was determined to calculate the copy number. A dilution series of each gene (10^8-10^2 copies) was used as a cDNA standard for the real-time PCR. The threshold cycle (C_t) in the PCR cycle at which fluorescence exceeds background was then converted to copy number based on a cDNA standard curve generated. Each experiment was carried out in duplicate.

Treatment with 5-aza-2′-deoxycytidine and/or trichostatin A

pNT1A, DU145, PC3, and LNCaP cells were seeded at 5×10^5/100-mm tissue culture dish. After 24 hours of incubation, the culture medium was changed to medium containing 5-aza-2′-deoxycytidine (5′-aza-dC; 5 μmol/L) for 96 hours and/or trichostatin A (TSA; 250 nmol/L)

Published OnlineFirst July 6, 2010; DOI:10.1158/1078-0432.CCR-09-3342

for an additional 24 hours. Total RNA extracted from cells and tissues using TRIzol reagent (Invitrogen) was first used in first-strand DNA (cDNA) synthesis using the Invitrogen SuperScript first-strand synthesis system and then used in real-time quantitative PCR as previously described (13). Mock-treated cells were cultured similarly.

Data analysis

The methylation index at each gene promoter and for each sample was calculated as the average value of mC/(mC + C) for all examined CpG sites in the gene and expressed as the percentage of methylation. Statistical significance was judged by the appropriate Mann-Whitney *t* test, Student *t* test, Pearson correlation, and simple and multiple regression methods used to compare the categorical variables of methylation changes in cancer by race and cancer × race interactions, or methylation changes in normal prostate tissues by race, age, and race × age interaction (summary in Supplementary Table). We used a cutoff corresponding to average methylation in normal

Table 2. Primer sequences used in the pyrosequencing analysis

Pyrosequencing			
	Sense	**Antisense**	**Sequencing primer**
GSTP1 (1st step)	AAGGAGGTTAGGG GTAAAAGTTATA	CCAAAACCTCCCCAATAC	GGGGGAGGATGTTAAG
GSTP1 (2nd step)	GAGTTAGGGGGAGGATGT	5′-Biotin-CCAAAACCTCCCCAATAC	
AR (1st step)	TAGGAAGTAGGGG TTTTTTAGGGTTAG	ACCCAACCCACCTCCTTACCT and U-ACCTCCCCTTTCCTTTTCTCC	TTGTTTTTTTAAA GTTATTAGGTA
AR (2nd step)	GTAGGGGTTTTTTA GGGTTAGAGTTAG	5′-Biotin-U	
RARβ2 (1st step)	AGTTGGGTTATT TGAAGGTTA	TACCCAAACAAACCCTACTC and U-CCCAAACAAACCCTACTC	GGGACACCGC TGATCGTTTA
RARβ2 (2nd step)	AAGTAGTAGGAAGT GAGTTGTTTAGA	5′-Biotin-U	
SPARC (1st step)	GGTGTAATTATAGAAG GGAAAGGTTGGG	U-CCTATTACCTATCTCTAAAC CCCTCCACATT	
SPARC (2nd step)	AAGGTTGGGAGGGGGTTA TATATATTTTAG	5′-Biotin-U	TTAGGGTAGTT TGAAGGAT
TIMP3 (1st step)	GGTGGGTGGGTG TTAGTTGG	U-CAAACCCTCCTACCCCTTCTC	
TIMP3 (2nd step)	GGTTTTGGTTTGGGT TAGAGATA	5′-Biotin-U	GGTTAGAGATATT TAGTGGTTTA
NKX2-5 (1st step)	GAGAGTAGGGTTG GGGAATATG	AACCCCTAACCCAATAACAAACT and U-CAATAACAAACTAAATCC CCCTCCTCTA	GGTAGTTTTTT TGTATGGTG
NKX2-5 (2nd step)	TAAGGTTTTTGGTAGTT TTTTTGTATGG	5′-Biotin-U	

RT-PCR			
	Forward	**Reverse**	**PCR size (bp)**
SPARC (STD)	TGTGCGAGCTGGATGAGAAC	GATGTACTTGTCATTGTCCAGGTC	599
SPARC	TGTGCGAGCTGGATGAGAAC	GTGGCAGGAAGAGTCGAAGG	128
TIMP3 (STD)	CCAAGGTGGTGGGGAAGAAG	AGCGGGAAGGGAGGGAAG	547
TIMP3	GGGGAAGAAGCTGGTAAAGGAG	AGCAGGTACTGGTACTTGTTGAC	171
NKX2-5 (STD)	GCCGCCTTCAAGCCAGAG	GCCCCACCAGCTCCAGAG	474
NKX2-5	CACGTCCACGCAGGTCAAG (TaqMan probe: CTGGTCCTG CCGCTGCCGCTTG)	GCCCCACCAGCTCCAGAG	88

NOTE: Primers used in the RT-PCR analysis are also shown, where STD represents the primers used to generate cDNA standards. U represents universal primer sequence—GGGACACCGCTGATCGTTTA.

Published OnlineFirst July 6, 2010; DOI:10.1158/1078-0432.CCR-09-3342

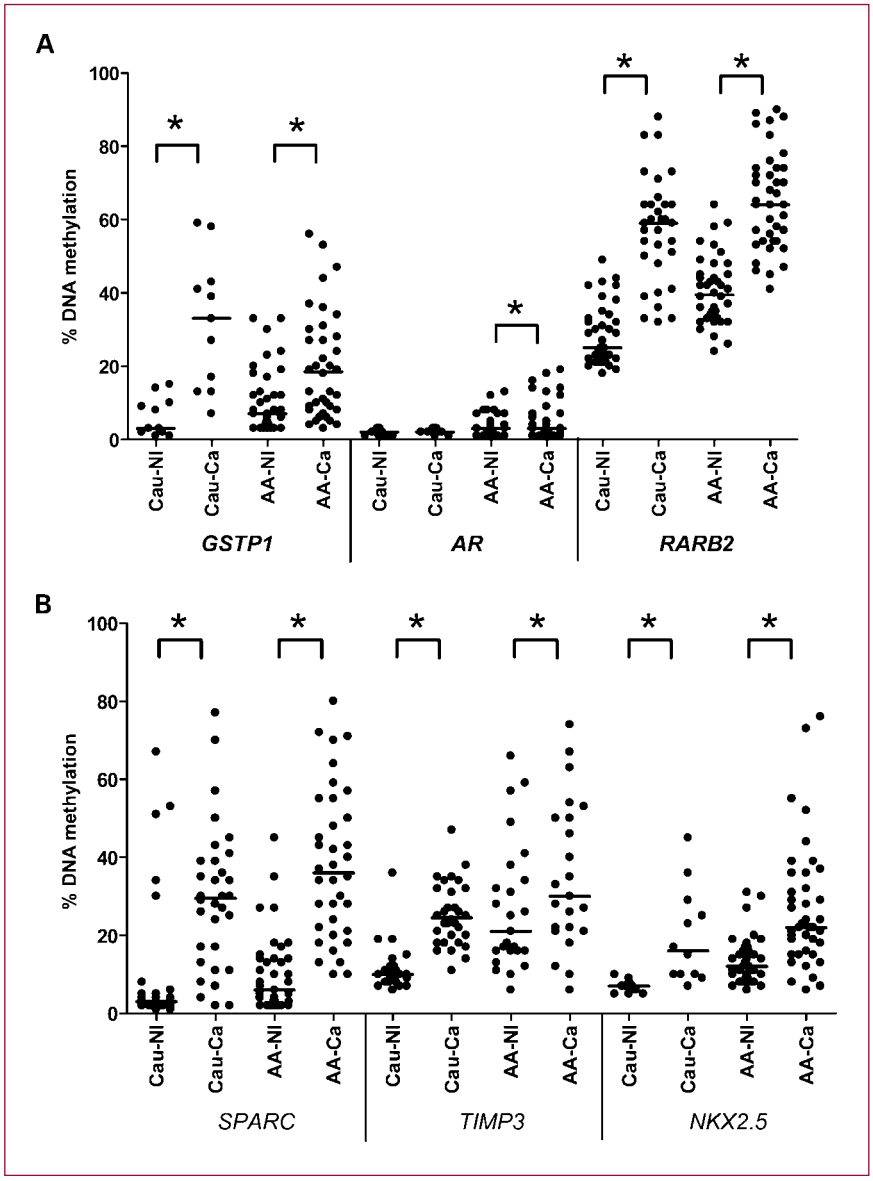


Fig. 1. Quantitative DNA methylation analysis in human prostate tissues. The percent DNA methylation levels of promoter CpG islands were analyzed in bisulfite-modified genomic DNA extracted from matched pairs of normal (Nl) and PCa (Ca) tissue samples obtained from AA and Cau cancer patients who had undergone radical prostatectomy. *Y* axis, percentage of methylated cytosines in the samples as obtained from pyrosequencing. *X* axis, normal and PCa tissues obtained from AA and Cau. *, $P < 0.05$ (Mann-Whitney *t* test). A, quantitative methylation analysis for *GSTP1, AR*, and *RARβ2*. B, quantitative methylation analysis for *SPARC, TIMP3*, and *NKX2-5*.

tissues + 2 SD to call a cancer as methylation positive (above the cutoff). The sensitivity, specificity, and accuracy of each individual or two combined candidate biomarkers were calculated. Sensitivity was defined as the number of true-positive (methylated in cancer) cases divided by the number of true-positive plus false-negative (not methylated in cancer) cases, and specificity was defined as the number of true-negative (not methylated in normal tissue) cases divided by the number of true-negative plus false-positive (methylated in normal tissue) cases. The accuracy of the test is measured by the area under the receiver operating characteristic (ROC) curve. Data analysis was done using either Prism 4 software (GraphPad Software, Inc.) or SPSS for Windows (version 13.0, SPSS). Significance was set at $P < 0.05$.

Results

We investigated the DNA methylation status for a panel of genes in matched normal and PCa tissue samples from radical prostatectomy specimens obtained from AA and Cau males with PCa to determine the prevalence of methylation changes in the two groups. We analyzed a total of six genes, including *GSTP1*, *AR*, and *RARβ2*, which we and others have previously shown to be hypermethylated in PCa tissue samples from Cau males (4, 14, 15); in addition, we also analyzed *SPARC, TIMP3*, and *NKX2-5*, which we have identified as being hypermethylated in the gene promoter using methylated CpG island amplification coupled with representational difference analysis in PCa cell lines (16). We analyzed the methylation status of

Published OnlineFirst July 6, 2010; DOI:10.1158/1078-0432.CCR-09-3342

these genes in 40 DNA samples from matched normal and PCa tissue samples obtained from AA males and between 12 and 40 DNA samples from matched normal and PCa tissue samples obtained from Cau males who underwent radical prostatectomy for PCa (age range, 52-75 years). Table 1 shows a summary of patient clinical data for the two groups, with Cau samples showing a nonsignificant slightly higher Gleason score. For each gene studied, the percentage of methylation at a specific promoter was compared between the matched normal and PCa tissue samples (Fig. 1). There was considerable variation in the percentage of CpG island methylation in the individual patient samples studied, presumably reflecting both random variability in tissue composition and variable methylation level per cell. The variable range of methylation could also reflect differences in genetic susceptibility to methylation, lifestyle, or other environmental exposures (including diet) and the random nature of the methylation event. The results showed significant hypermethylation for *GSTP1* ($P = 0.0001$ for AA; $P = 0.0008$ for Cau), *RARβ2* ($P < 0.001$ for AA and Cau), *SPARC* ($P < 0.0001$ for AA and Cau), *TIMP3* ($P < 0.0001$ for AA and Cau), and *NKX2-5* ($P < 0.0001$ for AA; $P = 0.003$ for Cau) in the PCa tissue samples when compared with the matched normal tissue samples. The *AR* gene showed low prevalence of methylation in the prostate tissue samples from both AA and Cau males. Regression analysis to examine whether methylation frequency in the normal and PCa tissue samples differed by race showed significantly higher methylation prevalence for *AR* ($P = 0.006$), *RARβ2* ($P < 0.0001$), *SPARC* ($P = 0.02$), *TIMP3* ($P < 0.0001$), and *NKX2-5* ($P = 0.002$) for AA in comparison with Cau samples, whereas PCa × race interaction showed significance for *GSTP1* ($P = 0.0069$; Supplementary Table). In addition, ROC analysis (Fig. 2) was done to compare the adjacent normal prostate and matched cancer tissues in AA, Cau, and both (Cau + AA) samples. The areas under the ROC curves were as follows: for *GSTP1*, 0.969 (Cau) and 0.811 (AA); for *AR*, 0.59 (Cau) and 0.531 (AA); for *RARβ2*, 0.969 (Cau) and 0.922 (AA); for *SPARC*, 0.75 (Cau) and 0.921 (AA); for *TIMP3*, 0.875 (Cau) and 0.668 (AA); and for *NKX2-5*, 0.984 (Cau) and 0.857 (AA). For the analysis in the CAU + AA samples, the areas under the ROC curves were 0.844 for *GSTP1*, 0.536 for *AR*, 0.925 for *RARβ2*, 0.878 for *SPARC*, 0.705 for *TIMP3*, and 0.884 for *NKX2-5*. The strength of the ROC curves indicates differences in the sensitivity of DNA methylated genes in the AA and Cau samples. Overall, the DNA methylation of *GSTP1*, *RARβ2*, *SPARC*, and *NKX2-5* genes indicates their potential as predictive genes for PCa detection in both AA and Cau male samples.

We next analyzed the methylation pattern in tissue punches of normal prostate collected from AA (18 samples; mean age, 62.7 ± 7.2 years) and Cau males (13 samples; mean age, 62.4 ± 8.8 years; $P = 0.89$; Table 1B). For each gene studied, the percentage DNA methylation at a specific promoter region was expressed as a function of age for both the AA and Cau male samples (Fig. 3). We observed methylation of the *GSTP1*, *RARβ2*, and *TIMP3* genes but this did not significantly correlate with age in both AA and Cau. The *SPARC* and *AR* genes were entirely unmethylated in the Cau male samples, although we observed some methylation in the AA samples. Regression analysis showed significantly higher methylation for *TIMP3* gene ($P = 0.039$) and *NKX2-5* gene ($P = 0.008$) in the AA samples when compared with the Cau samples.

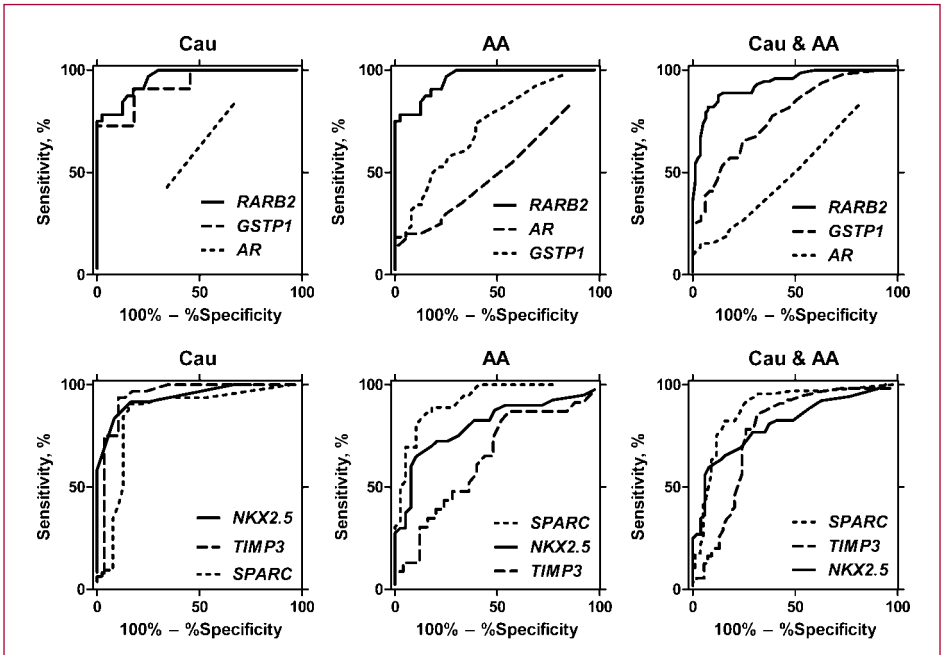


Fig. 2. ROC curves for methylation analysis in PCa tissue samples from AA, Cau, and both (AA + Cau). The areas under the ROC curves in Cau samples are 0.969 for *GSTP1*, 0.59 for *AR*, 0.969 for *RARβ2*, 0.75 for *SPARC*, 0.875 for *TIMP3*, and 0.984 for *NKX2-5*. The areas under the ROC curves in AA samples are 0.811 for *GSTP1*, 0.531 for *AR*, 0.922 for *RARβ2*, 0.921 for *SPARC*, 0.668 for *TIMP3*, and 0.857 for *NKX2-5*. The areas under the ROC curves in both Cau + AA samples are 0.844 for *GSTP1*, 0.536 for *AR*, 0.925 for *RARβ2*, 0.878 for *SPARC*, 0.705 for *TIMP3*, and 0.884 for *NKX2-5*.

Published OnlineFirst July 6, 2010; DOI:10.1158/1078-0432.CCR-09-3342

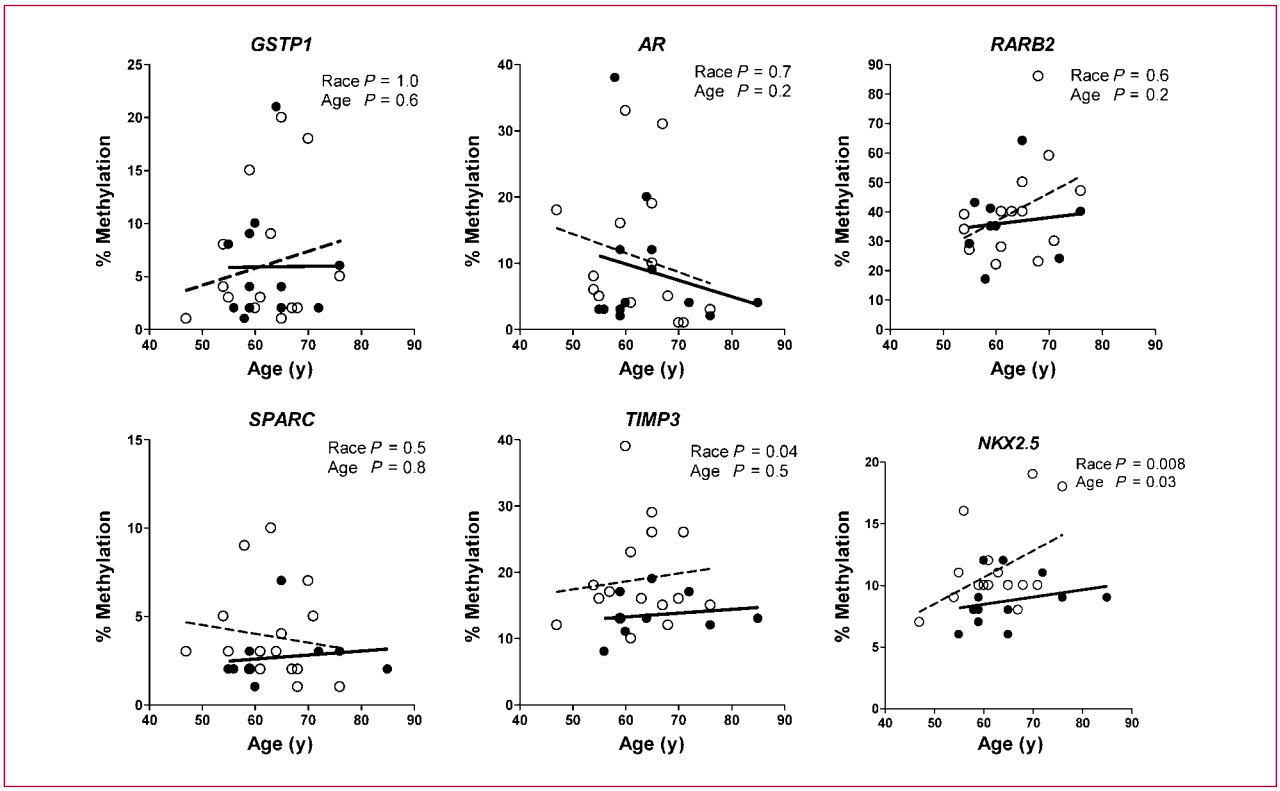


Fig. 3. Age-related DNA methylated gene profiles for AA (- - -) or Cau (—) men. CpG islands for *GSTP1*, *AR*, *RARβ2*, *SPARC*, *TIMP3*, and *NKX2-5* were analyzed in bisulfite-modified genomic DNA extracted from fresh-frozen tissue punches of normal prostate obtained from both AA and Cau. Eighteen samples from AA and 14 samples from Cau (age range, 47-85 y) were used. Regression analysis of the association of DNA methylation changes by race and age. *Y* axis, percentage of methylated cytosines in the samples as obtained from pyrosequencing. Each CpG island has a different scale range. *X* axis, age in years.

In addition, we observed a significant association between age and methylation level for *NKX2-5* gene ($P = 0.03$) in the AA samples when compared with the Cau samples. Furthermore, we observed a modest association of *NKX2-5* methylation ($P = 0.09$; Supplementary Table) for a race by age interaction. These results support the observation we made above of higher methylation of several genes in prostate tissue samples from AA in comparison with Cau. In addition, our data suggest that, for some genes, higher methylation differences maybe associated with aging.

To investigate if methylation is associated with the silencing of *SPARC*, *TIMP3*, and *NKX2-5* genes in PCa tissues, we performed expression analysis by quantitative RT-PCR. The results presented in Fig. 4 showed a significantly higher level of gene expression for *SPARC* ($P = 0.0213$) and *TIMP3* ($P < 0.0001$), but not *NKX2-5* ($P = 0.12$), in the matched normal prostate tissues when compared with the PCa tissues. Our data suggest an inverse association between DNA methylation and gene expression for *SPARC*, *TIMP3*, and *NKX2-5*. We observed that in cancer samples that showed higher methylation,

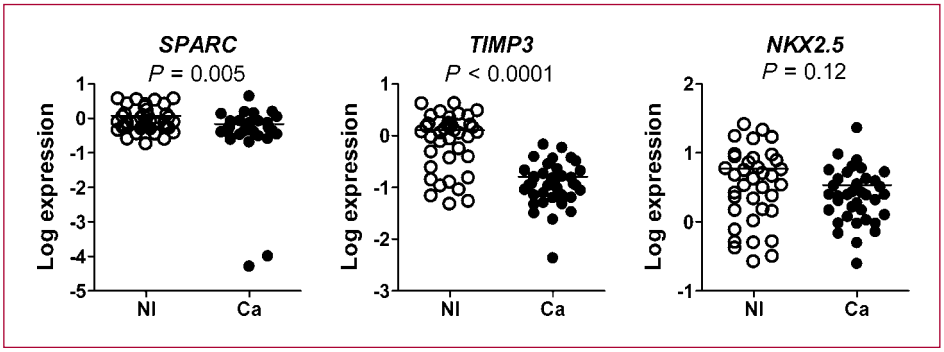


Fig. 4. Gene expression in prostate tissue samples. The relative mRNA transcript expression levels of *SPARC*, *TIMP3*, and *NKX2-5* were analyzed in 32 matched pairs of normal and PCa tissue samples by RT-PCR and expressed relative to β-actin to correct for variation in the amount of reverse-transcribed RNA. Relative expression (shown as log expression on the *Y* axis) was determined using gene copy number calculated from a standard curve.

Published OnlineFirst July 6, 2010; DOI:10.1158/1078-0432.CCR-09-3342

this was associated with low levels of gene expression, whereas the normal prostate samples had lower methylation and higher gene expression level to indicate that methylation leads to some loss of gene expression.

To verify that the expression of the hypermethylated genes can be restored, prostate cell lines were treated with the demethylating agent 5′-aza-dC, the histone deacetylase inhibitor TSA, or the combination of the two drugs, and gene expression was analyzed by quantitative RT-PCR (Fig. 5). The cell lines chosen for the analysis had high methylation levels for the genes tested and all had very low levels of expression at baseline. Results indicate at least a 2-fold increase in expression in response to 5′-aza-dC treatment for all genes analyzed in at least one cancer cell line, except for *TIMP3* gene, for which we did not detect any significant effect on expression level. The data support the observation we made above that silencing is related to DNA methylation. Treatment with the histone deacetylase inhibitor TSA alone did not restore gene expression for *SPARC*. On the other hand, *TIMP3* and *NKX2-5* showed increased expression after treatment with either TSA alone or the combination of 5′-aza-dC and TSA in at least one PCa cell line. The data show that both methylation and histone deacetylation seem to play a role in silencing the expression of *TIMP3* and *NKX2-5*, although methylation seems to play a dominant role in *NKX2-5* gene silencing.

Discussion

Ethnic differences in PCa incidence and mortality are well documented. The incidence and mortality for PCa is about 2-fold higher in AA than in Cau, with AA men experiencing among the highest rates worldwide (17). This disparity in PCa is believed to be a complex combination of environment and socioeconomic factors and genetics (for a recent review, see ref. 18). Several genetic variations in the human genome, particularly allelic variation in genes involved in pathways relevant to PCa biology, have been proposed as a genetic cause or contributor for the increased PCa risk in AA (19).

We have previously shown aberrant hypermethylation as a function of age in normal prostate tissues, which may precede and predispose to full-blown malignancy (4). Therefore, it may be possible to establish a quantitative cutoff point for the amount of methylation of some genes that would indicate the presence of cancer. In addition, the methylation prevalence may differ between ethnic groups such that methylated genes could also serve as ethnic-sensitive biomarkers for PCa detection. In the present study, we were interested in comparing the DNA methylation patterns in prostate tissue samples from AA and Cau. Previous work carried out by Enokida et al. (20) showed that *GSTP1* hypermethylation was significantly higher in PCa samples from AA in comparison with Cau and Asians. Other studies did not find significant difference in *GSTP1* methylation frequencies in PCa among AA and Cau (21, 22) but found higher frequency of CD44 hypermethylation in AA than in Cau (21). These studies had strictly focused on PCa tissues and benign prostatic hyperplasia (BPH) samples. However, our recent observations show that DNA methylation starts in normal prostate tissues as a function of age (4), and therefore, it is also important to analyze gene methylation in normal prostate samples from both AA and Cau. We therefore evaluated differences in DNA methylation of six genes, namely, *GSTP1*, *AR*, *RARβ2*, *SPARC*, *TIMP3*, and *NKX2-5*, which may play important regulatory roles in prostate disease etiology and/or progression. To our knowledge, our study is the first to analyze the methylation status of these genes in normal as well as matched pairs of normal and PCa tissue samples from AA and Cau males. Overall, we observed significant methylation prevalence in the PCa tissue samples from AA in comparison with Cau. The Cau cancer samples used in this study showed slightly higher Gleason score and similar pathologic staging when compared with the AA samples. Thus, the higher prevalence of methylation seen in the AA cancer samples is not simply reflective of differences in disease aggressiveness or stage between the two groups. In addition, regression analysis revealed significantly higher methylation for *NKX2-5*

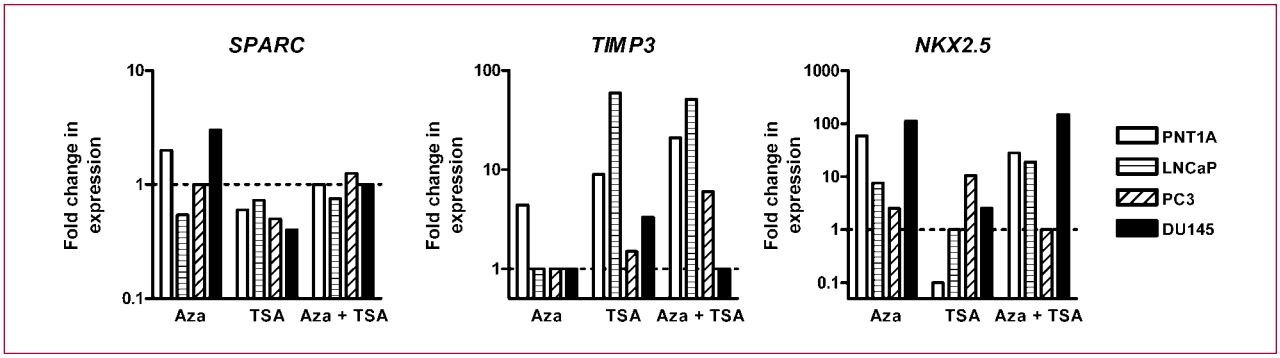


Fig. 5. Demethylation and gene expression. PCa cell lines and primary immortalized prostate epithelial cells, pNT1a, were treated with 5′-aza-dC (5 μmol/L), TSA (250 nmol/L), or a combination of the two drugs. The cell lines chosen for the analysis had high methylation levels for the genes tested and all had very low levels of expression at baseline. The fold change in gene expression relative to mock-treated cells was determined by quantitative RT-PCR and expressed relative to β-actin to correct for variation in the amounts of reverse-transcribed RNA. Points, average values of duplicate experiments.

Published OnlineFirst July 6, 2010; DOI:10.1158/1078-0432.CCR-09-3342

and *TIMP3* genes in the normal prostate tissue samples from AA in comparison with Cau. Of the six genes that we analyzed in the normal prostate tissue samples, methylation of *NKX2-5* also showed a significant association with age in AA in comparison with Cau. Our data suggest that not only is the methylation of *NKX2-5* suitable as a marker for PCa detection, but it may also have increased sensitivity for detecting PCa in AA. We have analyzed the relationship between DNA methylation changes and PCa risk but did not detect any significant association between DNA methylation changes and PCa risk by race. Although the sample size used in our studies is not large, the ROC analysis shows the differential predictive potential of DNA methylation of *GSTP1*, *RARβ2*, *SPARC*, *TIMP3*, and *NKX2-5* genes for PCa detection in AA and Cau samples. Thus, a larger PCa population size can confirm our observations and determine if the methylation status of these genes can provide a reliable and/or perhaps ethnic-sensitive index for the detection of PCa.

In summary, our panel of genes show significant differences in the prevalence of methylation in prostate tissue samples from AA and Cau. These genes play key regulatory roles in the pathways of prostate. Therefore, ethnic differences in the methylation pattern of these genes may contribute to the disparity associated with PCa. The genetic mechanism(s) underlying the differing prevalence of methylation in these two groups is an area of active investigation in our laboratory.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

DOD PCRP Idea Award PC073828; NIH/NCI grant CA091431 (B. Kwabi-Addo), the VA Merit Review program (M. Ittmann), NCI grant P50CA058204 to the Baylor Prostate Cancer Specialized Program of Research Excellence program, the NCI Supplement grant U01CA116937-05S2 (S.R. Patierno and N.H. Lee), and NIH Research Centers at Minority Institutions grant 2G12RR003048.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 12/23/2009; revised 05/07/2010; accepted 05/29/2010; published OnlineFirst 07/06/2010.

References

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
2. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042–54.
3. Nelson WG, Yegnasubramanian S, Agoston AT, et al. Abnormal DNA methylation, epigenetics, and prostate cancer. Front Biosci 2007;12:4254–66.
4. Kwabi-Addo B, Chung W, Shen L, et al. Age-related DNA methylation changes in normal human prostate tissues. Clin Cancer Res 2007;13:3796–802.
5. Hmadcha A, Bedoya FJ, Sobrino F, Pintado E. Methylation-dependent gene silencing induced by interleukin 1β via nitric oxide production21. J Exp Med 1999;190:1595–604.
6. Feinberg AP, Tycko B. The history of cancer epigenetics. Nat Rev Cancer 2004;4:143–53.
7. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.
8. Nelson WG, De Marzo AM, Isaacs WB. Prostate cancer. N Engl J Med 2003;349:366–81.
9. Bastian PJ, Yegnasubramanian S, Palapattu GS, et al. Molecular biomarker in prostate cancer: the role of CpG island hypermethylation. Eur Urol 2004;46:698–708.
10. Shu J, Jelinek J, Chang H, et al. Silencing of bidirectional promoters by DNA methylation in tumorigenesis. Cancer Res 2006;66:5077–84.
11. Colella S, Shen L, Baggerly KA, Issa JP, Krahe R. Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. Biotechniques 2003;35:146–50.
12. Polnaszek N, Kwabi-Addo B, Peterson LE, et al. Fibroblast growth factor 2 promotes tumor progression in an autochthonous mouse model of prostate cancer. Cancer Res 2003;63:5754–60.
13. Kwabi-Addo B, Wang J, Erdem H, et al. The expression of Sprouty1, an inhibitor of fibroblast growth factor signal transduction, is decreased in human prostate cancer. Cancer Res 2004;64:4728–35.
14. Ellinger J, Bastian PJ, Jurgan T, et al. CpG island hypermethylation at multiple gene sites in diagnosis and prognosis of prostate cancer12. Urology 2008;71:161–7.
15. Tokumaru Y, Harden SV, Sun DI, Yamashita K, Epstein JI, Sidransky D. Optimal use of a panel of methylation markers with GSTP1 hypermethylation in the diagnosis of prostate adenocarcinoma3. Clin Cancer Res 2004;10:5518–22.
16. Chung W, Kwabi-Addo B, Ittmann M, et al. Identification of novel tumor markers in prostate, colon and breast cancer by unbiased methylation profiling3. PLoS One 2008;3:e2079.
17. Hsing AW, Tsao L, Devesa SS. International trends and patterns of prostate cancer incidence and mortality. Int J Cancer 2000;85:60–7.
18. Freedland SJ, Isaacs WB. Explaining racial differences in prostate cancer in the United States: sociology or biology? Prostate 2005;62:243–52.
19. Robbins CM, Hernandez W, Ahaghotu C, et al. Association of HPC2/ELAC2 and RNASEL non-synonymous variants with prostate cancer risk in African American familial and sporadic cases. Prostate 2008;68:1790–7.
20. Enokida H, Shiina H, Urakami S, et al. Ethnic group-related differences in CpG hypermethylation of the GSTP1 gene promoter among African-American, Caucasian and Asian patients with prostate cancer16. Int J Cancer 2005;116:174–81.
21. Woodson K, Hayes R, Wideroff L, Villaruz L, Tangrea J. Hypermethylation of GSTP1, CD44, and E-cadherin genes in prostate cancer among U.S. Blacks and Whites33. Prostate 2003;55:199–205.
22. Woodson K, Hanson J, Tangrea J. A survey of gene-specific methylation in human prostate cancer among black and white men27. Cancer Lett 2004;205:181–8.

[Epigenetics 4:1, 54-61; 1 January 2009]; ©2009 Landes Bioscience

Research Paper

DNA methylation and aberrant expression of Sprouty1 in human prostate cancer

Bernard Kwabi-Addo[1,*] Chengxi Ren[2] and Michael Ittmann[2,3]

[1]Howard University Cancer Center; NW Washington DC USA; [2]Department of Pathology; Baylor College of Medicine; Houston, Texas USA; [3]Michael E. DeBakey Department of Veterans Affairs Medical Center; Houston, Texas USA

Key words: sprouty1, DNA methylation, pyrosequencing, prostate cancer, quantitative RT-PCR

Sprouty1 is a negative regulator of fibroblast growth factor signaling with a potential tumor suppressor function in prostate cancer (PCa). Sprouty1 is downregulated in human PCa and Sprouty1 expression can markedly inhibit PCa proliferation in vitro. The aim of this study was to investigate the role of DNA methylation in Sprouty1 expression in human prostate tumors. We used pyrosequencing to quantitatively measure the methylation status of the *Sprouty1* promoter region in prostate tissues and cell lines and assessed Sprouty1 mRNA expression by quantitative RT-PCR. Our data demonstrates significantly higher % methylation of Sprouty1 promoter in the PCa tissues when compared to matched normal tissues. Hypermethylation of *Sprouty1* promoter was detected in PCa cell lines compared to the normal prostate epithelial cells. The increased % methylation was associated with reduced Sprouty1 mRNA expression in the PCa tissues and cell lines. Methylation modification of the *Sprouty*1 promoter using *Sss*1 methylase abolished promoter activity whereas global demethylation with 5'-Aza-2'-Deoxycytidine treatment induced Sprouty1 expression. Our data demonstrates that DNA methylation in the *Sprouty1* promoter region is responsible for downregulating Sprouty1 expression in prostate cancer.

Introduction

Prostate cancer (PCa) is the second most common malignancy and the second leading cause of cancer deaths among men in the United States. There is abundant evidence to indicate that inappropriate activation of fibroblast growth factor receptor (FGFR) signaling plays a critical role in the initiation and progression of prostate cancer (reviewed in ref. 1).[1] Sprouty was originally identified in Drosophila as a negative regulator of fibroblast growth factor (FGF) signaling during tracheal development.[2] Subsequent studies have shown Sprouty to be a general inhibitor of growth factor-induced receptor tyrosine kinase (RTK) signaling pathways involved in Drosophila development and organogenesis.[3-5] While Drosophila has only one Sprouty gene, at least four Sprouty homologues (Sprouty1-4) have been found in humans and mice.[6,7] Mammalian Sprouty inhibit growth factor-induced cell responses by inhibiting the RTK-dependent Ras/mitogen-activated protein (MAP) kinase signaling pathway.[8-14] Several mechanisms for Sprouty inhibition of the RTK/Ras/MAP kinase pathway have been proposed, including blocking the interaction of the Grb2/SOS complex with the docking protein FRS2[3,15] or the inhibition of Raf.[11,12] Another characteristic of the Sprouty inhibitors is their regulation by growth factors in a negative feedback loop. Specifically, growth factors regulate both the level of Sprouty transcript[7] and in some systems, the recruitment of Sprouty proteins to the plasma membrane.[16] Given that Sprouty proteins can inhibit FGF signaling, they can potentially decrease the biological activities of FGFs in prostate cancer cells and inhibit their ability to promote cancer progression.

We have previously shown by immunohistochemical and quantitative real-time PCR analysis that Sprouty1 and Sprouty4 are downregulated in a subset of prostate cancers tissues when compared with normal prostate tissues.[17,18] McKie et al.[19] have observed that Sprouty2 expression is reduced in clinical prostate cancer tissues when compared with benign prostatic hyperplasia (BPH). These results were confirmed by Fritzsche et al.[20] In addition, downregulation of Sprouty transcripts was associated with biochemical recurrence following radical prostatectomy, indicative of more aggressive disease. The decrease in Sprouty expression in the human prostate cancer, despite elevated levels of FGF ligands and FGF receptors, implies a loss of an important growth regulatory mechanism in prostate cancers that may potentiate the effects of increased FGF and FGFR expression in prostate cancer tissues and may represent a novel mechanism that facilitates aberrant RTK signaling in prostate carcinogenesis.

We and others have also shown epigenetic inactivation to be a key mechanism for silencing Sprouty proteins in the prostate. For instance, we have observed promoter methylation at Sprouty4 CpG islands in prostate cancer. More than half of all prostate cancer tissue DNAs were methylated in this region and methylation significantly correlated with decreased Sprouty4 expression. Furthermore the treatment of prostate cancer cells with 5-aza-dC reactivated Sprouty4 expression demonstrating that aberrant methylation represents a key mechanism of Sprouty4 downregulation.[18] Similarly, extensive methylation of Sprouty2 has been observed in high grade invasive prostate cancers while control BPH tissues were predominantly

*Correspondence to: Bernard Kwabi-Addo; Howard University Cancer Center; 2041 Georgia Ave.; NW Washington DC 20060 USA; Tel.: 202.806.4092; Fax: 202.667.1686; Email: bkwabi-addo@howard.edu

Submitted: 10/31/08; Accepted: 11/20/08

Previously published online as an *Epigenetics* E-publication:
http://www.landesbioscience.com/journals/epigenetics/article/7400

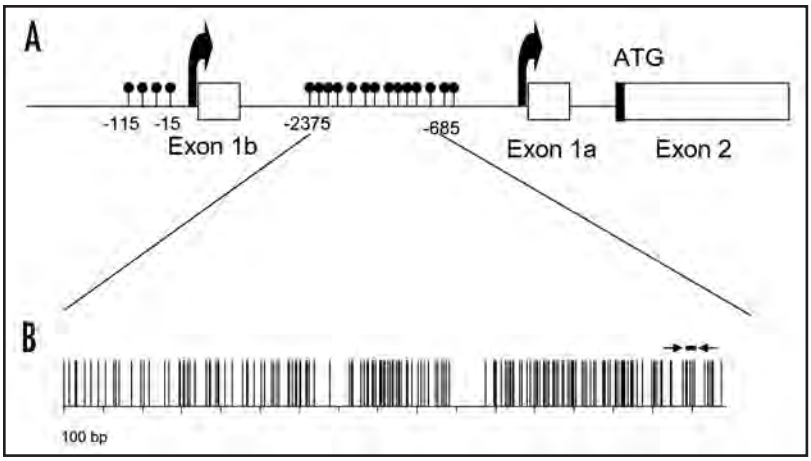


Figure 1. Genomic organization of the human *Sprouty*1 gene. (A) Exons are shown as open-boxes and translational start site, ATG is shown as a thick black bar. Promoter region is shown as blackened arrows. The use of alternative promoters does not result in protein isoforms because the variant 5' initial exons are joined to a common second exon that contains the translation initiation site. CpG dinucleotides are shown as short vertical lines with black circles on top. (B) Map represent CpG island upstream of Exon1a. Short vertical bars indicate CpG dinucleotides. Inverted arrows indicate PCR primers used in pyrosequencing assay. Horizontal black box indicate CpG region analyzed by pyrosequencing.

unmethylated.[19] The suppressed Sprouty2 expression correlated with methylation of the CpG region in clinical samples indicating that methylation of the Sprouty2 promoter was the likely cause of its transcriptional inactivation in the prostate. However, Fritzsche et al.[20] have reported low frequency of methylation of Sprouty2 promoter in prostate cancer cell lines and prostate cancer tissues. Furthermore, promoter methylation does not seem to explain Sprouty2 inactivation in breast cancer. Cultured breast cancer cell lines in the presence of 5'Aza-2-deoxycytidine (5-aza-dC) a demethylation agent, did not reactivate the expression of Sprouty2 and only minimal and patient specific methylation of the Sprouty2 CpG islands was found indicating other cancer-specific mechanisms independent of DNA methylation for Sprouty2 downregulation.[21]

The *Sprouty1* promoter contains a CpG island, and DNA methylation events that affect promoter activity offers a likely mechanism for epigenetic alteration in prostate cancer. Thus, in the present study, we sought to investigate the epigenetic mechanisms regulating Sprouty1 expression in prostate cancer.

Results

Genomic organization of the Sprouty1 gene. The human *Sprouty*1 transcript consists of two splice variants, 1a[22] and 1b[2] arising from two alternative promoters that map to human chromosome 4q27-28 and 4q25-28 respectively. Each splice variant has two exons and one intron. Exon 1 encodes the 5'-untranslated region of the cDNA, whereas exon 2 encodes the remainder of the 5'-untranslated region, the entire open-reading frame and the entire 3'-untranslated region. While the splice variants share the same second exon, they have different first exons, located very close to each other on the same chromosome (Fig. 1). The use of alternative promoters does not result in protein isoforms because the variant 5' initial exons are joined to a common second exon that contains the translation initiation site. In order to identify the transcription start sites of *Sprouty*1a and *Sprouty*1b splice variants, we performed 5'-RACE using poly $(A)^+$ RNA from fetal human lung and a *Sprouty*1 specific primer. We observed multiple bands after amplification, the largest of about 275 bp (data not shown). Sequence analysis identified multiple transcription initiation sites within the region -315 to -305 nucleotides from the first ATG codon in a Kozak consensus sequence. The 5'-most start site found is located at nucleotide position 160026 of the published sequence (GenBank accession no. AC026402). Because this region corresponds to the 5'-UTR of Splice variant 1b, this may represent the corresponding promoter region. Using similar approach we identified the transcription start site for Splice variant 1a to be at nucleotide position 162754 in the same published sequence (GenBank accession no. AC026402).

Functional characterization of Sprouty1 promoter region. To localize the DNA elements that are important for promoter activity, we carried out a series of unidirectional deletion analyses of up to 2 kb and approximately 1 kb of the 5'-flanking region of *Sprouty*1a of *Sprouty*1b splice gene variants, respectively. Deletion fragments were generated by PCR and cloned into the promoterless pGL3-Basic, a luciferase reporter vector. Each resulting recombinant construct was then transiently transfected along with the internal control pSV β-galactosidase plasmid into prostate cancer cell lines; LNCaP, PC3 and DU145 and the immortalized normal prostate cell line pNT1A. After 48 h, cell extracts were prepared and luciferase activity was measured and normalized to β-galactosidase activity. As shown in Figure 2, the promoter activities demonstrated significant difference between Splice 1a (Fig. 2A) and 1b (Fig. 2B) variants. *Sprouty*1a promoter strength was between 2- to 5-fold above the basal level. Whereas *Sprouty*1b promoter activity was between 40- and 900-fold above basal level depending on the cell line. Furthermore, the reporter gene expression levels showed significant differences among the different prostate cell lines suggesting that cell-specific element(s) may be present in these sequences. Interestingly, the androgen-dependent cell line, LNCaP which expressed the least Sprouty1 protein level as determined by western blot analysis[17] showed the strongest promoter activity; expressing over 7-fold higher promoter activity than any of the other cell lines. Overall, we observed maximum promoter activity for Splice 1a and 1b variants in the promoter fragments pSprouty1aFwd5 and pSprouty1bFwd3 respectively, in all the cell lines investigated. Because the activity of the *Sprouty*1b promoter region was over 100-fold higher than that of Sprouty1a, we believe that *Sprouty1b* region has the transcriptional elements and or enhancer sequence(s) necessary for *Sprouty*1 gene regulation. Whereas *Sprouty1a* promoter activity maybe regulated by other phenomenon such as epigenetic events.

Global DNA methylation analysis using the *Sss* I methylase assay. Using the MethPrimer software package for CpG islands identification (www.urogene.org/methprimer/), we have identified two separate CpG islands: 1 spanning about 1.7 kbp of the *Sprouty*1a promoter region and the other spanning about 100 bp of *Sprouty*1b promoter region (as shown in Fig. 1). Using a series of unidirectional PCR based deletion analysis followed by luciferase reporter assay, we have identified optimal promoter activity for *Sprouty*1a Fwd5 and *Sprouty*1b Fwd3 (shown in Fig. 2) and these are here

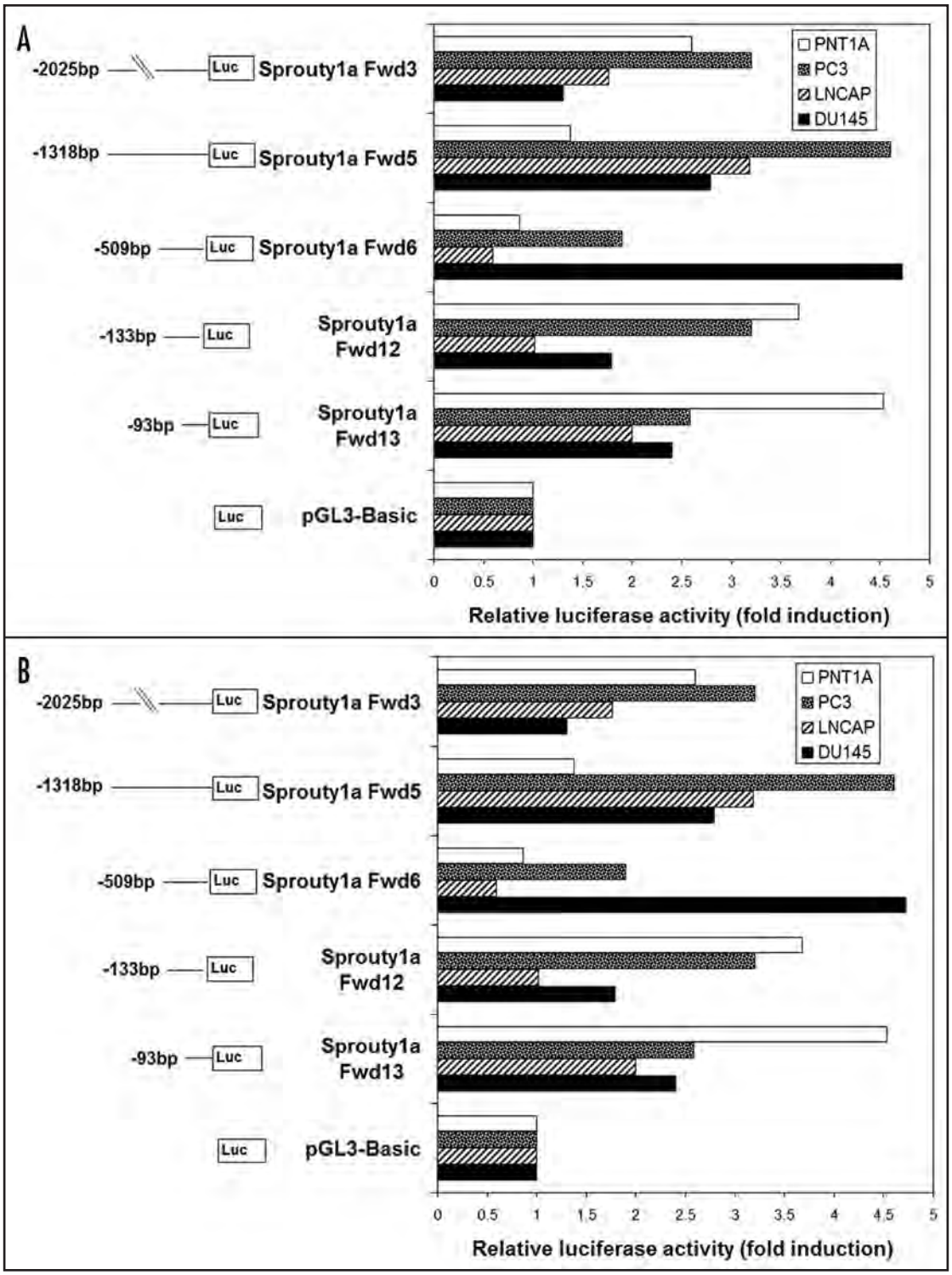


Figure 2. Progressive deletion analysis of the 5'-flanking region of splice variant 1a and 1b of the human *Sprouty*1 gene. The schematic diagrams represent a series of *Sprouty*1a (A) and 1b (B) gene constructs with variable 5'-ends as indicated. The luciferase activity was measured and normalized for transfection efficiency by dividing the measurement of the firefly luciferase activity by that of the β-galactosidase activity. The relative luciferase activities are represented as fold induction with respect to that obtained in cells transfected with the empty control vector (pGL3-Basic). Data represents the mean of triplicate experiments.

after referred to as *Sprouty*1a and *Sprouty*1b promoter respectively. To investigate whether constitutively active *Sprouty*1a and/or *Sprouty*1b promoter activity was inhibited by the methylation of the promoter CpG island, we modified the promoter constructs with *Sss*I methylase treatment and examined the activity of the methylated promoter. When the *Sss*I methylated or non-methylated *Sprouty*1a and *Sprouty*1b promoter constructs were each transiently transfected into LNCaP cells the activity of the methylated *Sprouty*1a promoter

was only 5% of that of the unmethylated construct (Fig. 3). On the other hand, CpG methylation of *Sprouty*1b construct did not show significant effect on its activity when compared to the control unmethylated construct (Fig. 3). This observation indicates that methylation of the *Sprouty*1a promoter may be involved in the control of Sprouty1 expression.

In vitro methylation analysis of the sprouty1 promoter region. To investigate whether *Sprouty*1a promoter is methylated in human prostate tumors, we used pyrosequencing to quantitatively measure DNA methylation of bisulfite modified genomic DNA. Typical examples of bisulfite methylation profiles at thee CpG sites of the *Sprouty*1 promoter is presented as pyrogram for the immortalized normal prostate epithelial cell line; pNT1A and the prostate cancer cell lines; LNCaP, PC3 and DU145 (Fig. 4A). As shown in the pyrogram, the pNT1A cells demonstrated on average 5% methylation at each CpG site. On the other hand, LNCaP, PC3 and DU145 cells demonstrated an average of 30%, 15% and 20% methylation respectively, at each CpG site indicating increased methylation of *Sprouty*1 in LNCaP, PC3 and DU145 cells but low methylation in pNT1A cells. Interestingly, pNT1A cells expressed the highest levels of Sprouty1 mRNA expression whereas LNCaP, PC3 and DU145 cells showed much lower levels of the Sprouty1 mRNA expression as determined by quantitative RT-PCR analysis (Fig. 4B). The inverse association between Sprouty1 mRNA expression and % DNA methylation level suggests that DNA methylation maybe a mechanism for downregulating Sprouty1 expression in the prostate cancer cell line.

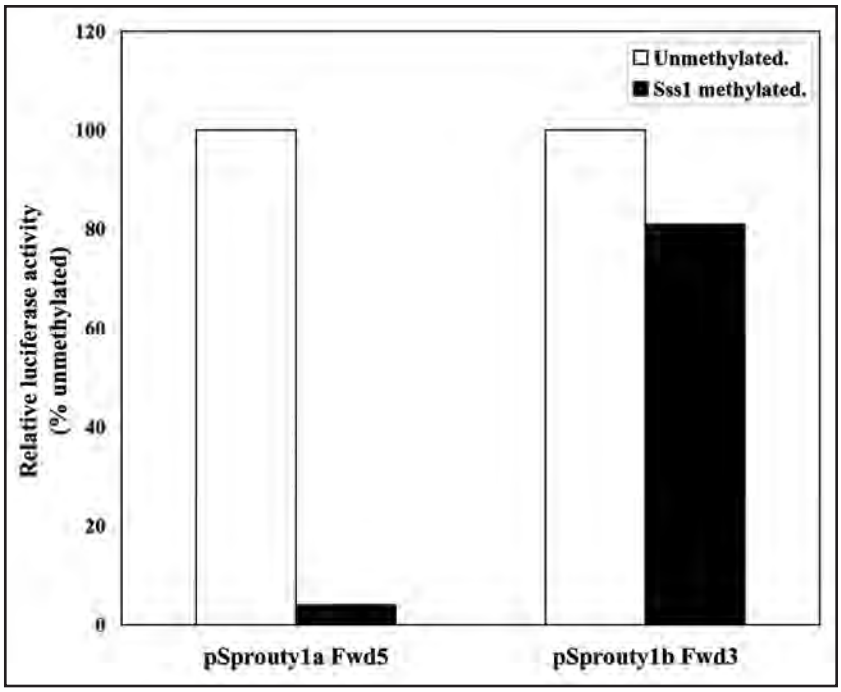


Figure 3. Effect of in vitro methylation and *Sprouty*1 promoter activity. *Sprouty*1a (p*Sprouty*1a Fwd5) and 1b (p*Sprouty*1b Fwd3) promoters were methylated in vitro by *SssI* methylase. Methylated and unmethylated *Sprouty*1a and 1b promoters were transfected into LNCaP cells and assayed for luciferase activity. The luciferase activity was measured and normalized for transfection efficiency by dividing the measurement of the firefly luciferase activity by that of the β-galactosidase activity. The relative luciferase activities are represented as fold induction with respect to that obtained in cells transfected with the empty control vector (pGL3-Basic). Results are shown as percentages, with luciferase activity due to unmethylated promoter designated as 100%. Data represents the mean of triplicate experiments.

Next, we used pyrosequencing to quantitatively measure DNA methylation of bisulfite modified genomic DNA in matched normal and prostate cancer tissue samples from 15 prostate cancer patients. The average percentage of methylation at the three CpG sites of the *Sprouy1* promoter was compared between the matched normal and prostate cancer tissue for each patient (Table 1). Our data showed approximately 1.6-fold higher % methylation level in the prostate cancer tissues (7.0 ± 0.619, SEM) when compared with the matched normal prostate tissues (4.33 ± 0.398, SEM). We next used quantitative RT-PCR to measure Sprouty1 mRNA in these same prostate tissue samples. We found that Sprouty1 expression was approximately 1.8-fold higher in the normal prostate tissues (9.78 ± 1.99, SEM) compared to prostate cancer tissues (5.31 ± 1.294, SEM). The reduced Sprouty1 mRNA expression in the prostate cancer tissues was associated with increased % DNA methylation in the prostate cancer tissues compared to the normal prostate tissues. This data indicates that the reduced Sprouty1 expression in prostate cancer tissues may reflect an increased in promoter methylation in this same tissue samples.

Treatment of prostate cancer cell lines with 5'-Aza -2'deoxycytidine can restore Spry1 expression. To further investigate whether DNA methylation plays a role in Sprouty1 expression, we tested the hypothesis that pharmacological modulation of methylation can reactivate gene expression.[23] To achieve this we treated pNT1A, DU145, PC3 and LNCaP cells in various doses of the DNA methyltransferase inhibitor 5-aza-2'deoxycytidine (5-aza-dC). As shown in Figure 5, treatment of the prostate cancer cell lines, DU145, PC3 and LNCaP with 5-aza-dC (2 μM) led to a significant increase in Spry1 mRNA expression in the prostate cancer cell lines. Taken together, these data suggests that promoter methylation may play a role in downregulating Sprouty1 expression in these cell lines and human prostate tumors.

Discussion

In the present study, we have investigated DNA methylation and Sprouty1 expression in human prostate cancer. DNA methylation is a common event in cancer and several genes promoter methylation has been reported. For example, aberrant methylation of GSTP1 gene is perhaps the most common genomic alteration in human prostate cancer and occurs in the earliest stages of prostate carcinogenesis.[24] Because of the presence of a large CpG island (1.7 kbp) of the *Sprouty1* promoter region, we speculated that DNA methylation may contribute to the silencing of Sprouty1 expression. We used pyrosequencing to quantify the methylation status of three CpG sites in the *Sprouty*1 promoter. Pyrosequencing offers a semi-quantitative high-throughput and reliable method with an in built internal control for adequacy of bisulfite treatment.[25] Using this approach, we have observed methylation in normal as well as prostate cancer tissues analyzed. It is not unusual to detect DNA methylation in normal prostate tissues as we have recently

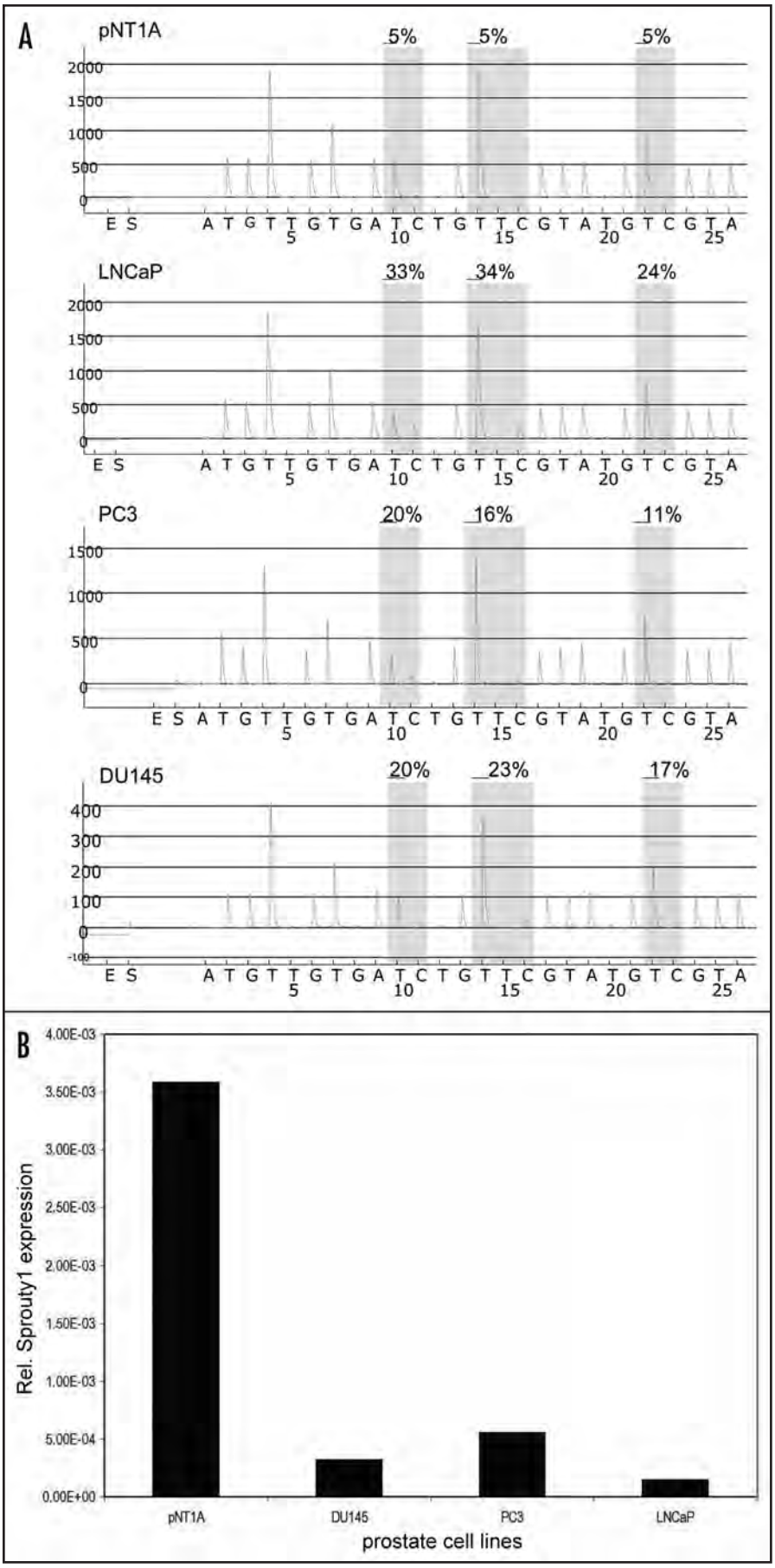


Figure 4. Methylation and expression analysis of the Sprouty1. (A) Representative program traces for *Sprouty*1. Gray colums represents regions of C to T polymorphic sites. Genomic DNA sample extracted from immortalized normal prostate epithelial cell line, pNT1A cells, and genomic DNA sample extracted from prostate cancer cell lines LNCaP, PC3 and DU145 are shown. Top, percentage of methylation at each CpG site. Y-axis; signal peaks proportional to the number of nucleotides incorporated. X-axis; the nucleotide sequence incorporated. (B) The Sprouty1 mRNA expression in normal prostate and prostate cancer cell lines as determined by quantitative reverse transcription-PCR. The Sprouty1 expression was assessed by quantitative RT-PCR using a real-time thermal cycler (iCycler; Bio-Rad). Sprouty1 expression levels are displayed as a ratio of Sprouty1 transcripts x 10^3 to β-actin transcripts. The data is a representative of duplicate experiments.

demonstrated that DNA methylation changes starts in the normal prostate tissue as a function of age and this dramatically increases in prostate cancer.[26] The samples we used in our analysis are derived from patients 50 years of age and older so we would expect some degree of methylation in the normal prostate tissues. On average, we observed a significantly higher methylation in the 15 prostate cancer cases when compared to the matched normal prostate tissues. However, because of the limited sample size that was analyzed, we were unable to observe direct statistical correlation between methylation and mRNA expression in our studies. Nonetheless, our observation of higher methylation in the prostate cancer cell lines prostate cancer tissues which showed reduced Sprouty1 expression compared to normal prostate cells and prostate tissues clearly indicate that methylation is responsible for reduced expression of Sprouty1 in prostate cancer. This observation is supported by our published data showing that methylation of *Sprouty*4 significantly correlated with decrease in Sprouty4 expression in prostate cancer.[18] This data clearly demonstrate methylation as a key mechanism for the inactivation of Sprouty genes in human prostate cancer.

However, in three cases where we detected low methylation in the cancer tissues compared to the normal tissues, we also observed low Sprouty1 expression. Other mechanisms of gene inactivation, such as alterations in trans-acting factors, and heterozygous or homozygous deletion could also affect Sprouty1 expression and this remains to be explored. The expression of Sprouty1 is driven by two promoters which are responsible for the synthesis of one transcript. The internal promoter has a very large CpG island and our studies demonstrate that methylation of this promoter site is responsible for decreased expression of Sprouty1 protein. The upstream promoter does not seem to

be affected by methylation, however, this region demonstrates strong promoter activity and may contain key regulatory binding sites for basal transcription. Whether additional mechanism(s) of gene inactivation, such as alteration in trans-acting factors is being actively investigated by our laboratory.

Materials and Methods

Cell culture. The human prostate cancer cell lines, PC3, DU145 and LNCaP were obtained from the American Type Culture Collection (Manassas, VA). The immortalized normal prostate epithelial cell line pNT1A was obtained from the European Collection of Cell Cultures (Salisbury, U.K). All cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) unless otherwise stated.

Human prostate tissue samples. All samples of human prostate tissues were obtained with informed consent and maintained by the Baylor Specialized Program of Research Excellence (SPORE) in the prostate cancer tissue bank.[27] Fresh frozen tissue punches of normal and tumor tissues were obtained at the time of radical prostatectomy. The pathological status was confirmed before processing, and the tumor samples had a tumor cell percentage of 70%–100% with Gleason scores of 6–8.

Bisulfite modification, PCR and pyrosequencing analysis of the Sprouty1 promoter. DNA samples prepared from prostate tissues and cell lines were modified by sodium bisulfite treatment using MethylEasy kit (Human Genetic Signatures, Sydney, AUS) according to the manufacturer's protocol.

Bisulfite PCR primers were designed based on bisulfite/converted sequence from the *Sprouty1* CpG island ensuring that the bisulfite-PCR primers avoid CpG sites and that they are designed as close to the transcription start site as possible. A two step PCR reaction was carried out using 2 μl of bisulfite converted genomic DNA and two sets of different bisulfite PCR primers in a standard PCR reaction. One of the primers (reverse primer) in the 2nd step PCR reaction was biotinylated in order to create a single-stranded DNA template for the pyrosequencing reaction. Primers used in the 1st step PCR reaction were forward 5'-AGG GTT TTT AGA GAG GAT AAT TTG GGT TAT-3' and reverse 5'-CCC CCA CTT CTA AAA ACT CAA AAT TAA ATA and a reverse primer tailed with a universal sequence (shown as underlined sequence) 5'-GGG ACA CCG CTG ATC GTT TAC CCC CAC TTC TAA AAA CTC AAA ATT AAA TA-3'. The reverse primer and the reverse primer tailed with the universal primer were mixed at a1:9 in the PCR reaction respectively. The primers used in the 2nd step PCR reaction were forward 5'-TTT AGG GTA ATA GGG GAT GGA GGA-3' and biotinylated universal primer 5'-GGG ACA CCG CTG ATC GTT TA-3'. Integrity of the PCR product was verified on 1.5% agarose gels with ethidium bromide staining. The product from the 2nd step PCR reaction was immobilized on streptavidin-sepharose beads (Amersham), washed, denatured and the biotinylated strands released into an annealing buffer containing the sequencing primer. Pyrosequencing was performed using the PSQ HS96 Gold SNP Reagents on a PSQ 96HS machine (Biotage). Each pyrosequencing reaction was done at least twice.

The PCR amplification step was as follows: 95°C for 3 min, then denature at 95°C for 30 sec, anneal at 54°C (1st step) or 58°C (2nd step) for 30 sec, extension at 72°C for 30 sec for 50 cycles, and a final 10 min extension at 72°C.

Construction of plasmids for promoter analysis. Progressive deletion constructs of *Sprouty*1a and *Sprouty*1b 5'-flanking regions were accomplished by unidirectional cloning of PCR fragments from the

Table 1 **Relationship between Sprouty1 mRNA expression (relative to β-actin expression) determined by quantitative RT-PCR and *Sprouty1* methylation in matched pairs of normal and prostate cancer tissues samples from 15 prostate cancer patients**

	Relative sprouty1 expression		% Methylation	
Patient #	Normal	Cancer	Normal	Cancer
1	13.8	9.84	3	6
2	26.7	3.66	4	10
3	8.10	2.97	3	9
4	1.06	0.783	8	5
5	5.30	0.697	4	7
6	10.0	5.25	5	10
7	10.8	9.48	3	4
8	5.44	2.65	3	7
9	24.4	6.63	3	3
10	2.23	0.194	6	12
11	9.33	5.29	4	8
12	12.8	15.1	4	4
13	1.06	1.75	6	7
14	12.8	15.1	6	5
15	3.0	0.343	3	8
Average	9.78	5.31	4.33	7.0

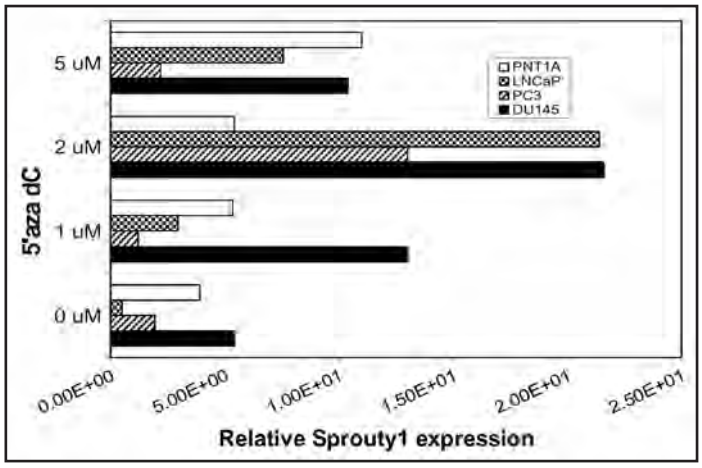


Figure 5. Demethylation and Sprouty1 expression. Prostate cancer cell lines; LNCaP, PC3 and DU145, and immortalized primary prostatic epithelial cells; pNT1A were each treated with 5'-aza-2'-deoxycytidine (5'-aza-dC) at the indicated concentrations for 96 hours. Sprouty1 mRNA expression was determined by quantitative RT-PCR using iCycler and expressed relative to β-actin to correct for variation in the amounts of reverse-transcribed RNA. The data is a representative of duplicate experiments.

*Sprouty*1 5'-flanking region into the *Kpn*1/*Nhe*1 site of the promoterless and enhancerless firefly luciferase reporter vector pGL3-Basic (Promega). For each region the PCR fragments were generated using a common reverse primer and different forward primers. The *Kpn1* and *NheI* sites (showed in lowercase) were engineered into the 5' ends of forward and reverse primers, respectively. The numbers indicated after the primer sequences correspond to the distance in nucleotides from the 5-end of the sequence in uppercase to the 5'-most transcription start site determined by 5'-RACE. For *Sprouty*1a 5'flanking region: Forward (Fwd) 13 (-93) 5'-ggt acc TCC TAC CAC AGA GAG AGG GAG AAA-3'; Fwd12 (-133) 5'-ggt acc CCC TCC TGA GCT CAT GGT AAC CT-3'; Fwd 6 (-509) 5'-ggt acc CTT CTG GTT TGG AGC ACA GTG CAA AG-3'; Fwd 5 (-1318) 5'ggt acc AGA AGA CCT CCC GAG GTG GAT GTT A-3'; Fwd3 (-2025) 5'-ggt acc CTG TCA ATC ACC GGG AGC-3'; Reverse (+8) 5'-gct agc AAT CCG CAC TGA ATA AAT AGT TGA C-3'. For *Sprouty*1b 5-flanking region: Fwd2 (-70) 5'-ggt acc CAT GAT ATC ACC GGA GGC GTG TCC TG-3'; Fwd3 (-175) 5'-ggt acc GAG TCT GTA GGG CAA CAT TTC CAA GTT GG-3'; Fwd 4 (-233) 5'-ggt acc CTG CAT TTG CAG AAT TTT TAG AGG CAC-3'; Fwd 5 (-305) 5'-ggt acc CAC CAA TCC TTT TAA TTG AGA TCG AC-3'; Fwd6 (-530) 5'-ggt acc CAT ATG CTT ATA TTA CAT TTG CAG TAA GG-3'; Fwd 7 (-960) 5'-ggt acc GTT TTG CCA GAC TTT AAG CTA CTC C-3'; Reverse (+50) 5'-gct agc GGG AAT GTG CTG ATA ATC ACT CG-3'. We used the Advantage Genomic PCR kit (Clontech) to amplify these promoter fragments and according to the manufacturer's protocol. The human genomic DNA was used as template. The PCR products were first cloned into TOPO TA vector (Invitrogen) and then excised by *Kpn*1 and *Nhe*1 digestion and subcloned into the pGL3-Basic vector. Every construct was sequenced to ensure correct orientation and sequence integrity.

Transient transfections. The LNCaP cells were seeded on a six-well tissue plates in RPMI-1640 medium and supplemented with 10% FBS and grown for 16–24 hours to 80% confluence. Next, cells were transiently transfected with the individual luciferase reporter plasmid by using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's procedure. The pSV-β-galactosidase control vector (Promega) was cotransfected with various luciferase reporter plasmids into cells to normalize the variations in transfection efficiency. Each transfection was done in triplicate.

Reporter gene luciferase assay. Cells were lysed 48 hours post-transfection by freeze thaw (three cycles) in luciferase reporter lysis buffer (Promega). The lysates were centrifuged at 12,000 g for 2 min to remove cell debris. The supernatant was used for both luciferase and β-galactosidase activity assays. Luciferase activity was determined by using a luciferase assay kit (Promega) according to the manufacturer's protocol and measured in a luminometer. The β-galactosidase activity was assayed using the β-galactosidase enzyme assay kit (Roche) according to the manufacturer' protocol. Variation in transfection efficiency was normalized by dividing the measurement of the firefly luciferase activity by that of the β-galactosidase activity. The promoterless pGL3-Basic vector was used as negative control, and the pGL3-CMV plasmid (which has CMV promoter and enhancer to drive the luciferase gene) was used as positive control for each transfection assay. Each reporter gene assay was done in triplicate.

In vitro methylation. The *Sprouty*1a Fwd5 and *Sprouty*1b Fwd 3 promoter fragments (1 μg) were in vitro methylated with *Sss*I (CpG) methylase (New England Biolabs, Beverly, MA) as recommended by the manufacturer. The *Sss*I methylation, which methylates all cytosine residues within the double-stranded dinucleotide recognition sequence (5'-CG-3'), was performed at 10 mM Tris, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 160 μM *S*-adenosylmethionine at 37°C for 1 h. After the methylation reaction the promoter fragments were purified by phenol chloroform extraction and ethanol precipitation. The methylated DNA fragments were then digested with *Nhe*1 and *Kpn*1 and then sub-cloned into the pGL3-Basic vector. The methylated promoter constructs were used for transient transfection assays. Individual reactions were monitored by digestion with *Sss*I or *Hpa*II or *Hha*I restriction enzymes.

Induction of sprouty1 expression by 5'-aza-2'-deoxycytidine (5'-aza-dC). The pNT1A, DU145, PC3 and LNCaP cells were seeded at 5 x 10^5 cells/100-mm tissue culture dish. After 24 hours of incubation, the culture media was changed to media containing 5'-aza-dC for 96 hours. Total RNA extracted from cells and tissues using TRIzol Reagent (Invitrogen) was first used in first strand DNA (cDNA) synthesis using Invitrogen Super-ScriptTM first strand synthesis and then used in real-time quantitative PCR as previously described.[17]

Conclusion

We have identified a 1.7 kbp CpG island of the human *Sprouty*1 gene promoter and DNA methylation of this promoter region appears to play a role in downregulating Sprouty1 expression in human prostate cancer.

Acknowledgements

The helpful comments of Dr. Jaroslov Jelinek (M.D. Anderson Cancer Center) are gratefully acknowledged. This work was supported by grant from the Department of Defense Prostate Cancer Research Program New Investigator Award; PC040326 to Bernard Kwabi-Addo: the Department of Veterans Affairs Merit Review program to Michael Ittmann and the National Cancer Institute to the Baylor prostate cancer SPORE program (P50CA058204).

References

1. Kwabi-Addo B, Ozen M, Ittmann M. The role of fibroblast growth factors and their receptors in prostate cancer. Endocr Relat Cancer 2004; 11:709-24.
2. Hacohen N, Kramer S, Sutherland D, Hiromi Y, Krasnow MA. sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. Cell 1998; 92:253-63.
3. Casci T, Vinos J, Freeman M. Sprouty, an intracellular inhibitor of Ras signaling. Cell 1999; 96:655-65.
4. Kramer S, Okabe M, Hacohen N, Krasnow MA, Hiromi Y. Sprouty: a common antagonist of FGF and EGF signaling pathways in Drosophila. Development 1999; 126:2515-25.
5. Reich A, Sapir A, Shilo B. Sprouty is a general inhibitor of receptor tyrosine kinase signaling. Development 1999; 126:4139-47.
6. de Maximy AA, Nakatake Y, Moncada S, Itoh N, Thiery JP, Bellusci S. Cloning and expression pattern of a mouse homologue of drosophila sprouty in the mouse embryo. Mech Dev 1999; 81:213-6.
7. Minowada G, Jarvis LA, Chi CL, Neubuser A, Sun X, Hacohen N, Krasnow MA, Martin GR. Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. Development 1999; 126:4465-75.
8. Hanafusa H, Torii S, Yasunaga T, Matsumoto K, Nishida E. Shp2, an SH2-containing protein-tyrosine phosphatase, positively regulates receptor tyrosine kinase signaling by dephosphorylating and inactivating the inhibitor Sprouty. J Biol Chem 2004; 279:22992-5.
9. Gross I, Bassit B, Benezra M, Licht JD. Mammalian sprouty proteins inhibit cell growth and differentiation by preventing ras activation. J Biol Chem 2001; 276:46460-8.
10. Yigzaw Y, Cartin L, Pierre S, Scholich K, Patel TB. The C terminus of sprouty is important for modulation of cellular migration and proliferation. J Biol Chem 2001; 276:22742-7.
11. Impagnatiello MA, Weitzer S, Gannon G, Compagni A, Cotten M, Christofori G. Mammalian sprouty-1 and -2 are membrane-anchored phosphoprotein inhibitors of growth factor signaling in endothelial cells. J Cell Biol 2001; 152:1087-98.

12. Sasaki A, Taketomi T, Kato R, Saeki K, Nonami A, Sasaki M, et al. Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. Nat Cell Biol 2003; 5:427-32.
13. Yusoff P, Lao DH, Ong SH, Wong ES, Lim J, Lo TL, et al. Sprouty2 inhibits the Ras/MAP kinase pathway by inhibiting the activation of Raf. J Biol Chem 2002; 277:3195-201.
14. Lee SH, Schloss DJ, Jarvis L, Krasnow MA, Swain JL. Inhibition of angiogenesis by a mouse sprouty protein. J Biol Chem 2001; 276:4128-33.
15. Hanafusa H, Torii S, Yasunaga T, Nishida E. Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. Nat Cell Biol 2002; 4:850-8.
16. Lim J, Wong ES, Ong SH, Yusoff P, Low BC, Guy GR. Sprouty proteins are targeted to membrane ruffles upon growth factor receptor tyrosine kinase activation. Identification of a novel translocation domain. J Biol Chem 2000; 275:32837-45.
17. Kwabi-Addo B, Wang J, Erdem H, Vaid A, Castro P, Ayala G, Ittmann M. The expression of Sprouty1, an inhibitor of fibroblast growth factor signal transduction, is decreased in human prostate cancer. Cancer Res 2004; 64:4728-35.
18. Wang J, Thompson B, Ren C, Ittmann M, Kwabi-Addo B. Sprouty4, a suppressor of tumor cell motility, is downregulated by DNA methylation in human prostate cancer. Prostate 2006; 66:613-24.
19. McKie AB, Douglas DA, Olijslagers S, Graham J, Omar MM, Heer R, et al. Epigenetic inactivation of the human sprouty2 (hSPRY2) homologue in prostate cancer. Oncogene 2005; 24:2166-74.
20. Fritzsche S, Kenzelmann M, Hoffmann MJ, Muller M, Engers R, Grone HJ, et al. Concomitant downregulation of SPRY1 and SPRY2 in prostate carcinoma. Endocr Relat Cancer 2006; 13:839-49.
21. Lo TL, Yusoff P, Fong CW, Guo K, McCaw BJ, Phillips WA, et al. The ras/mitogen-activated protein kinase pathway inhibitor and likely tumor suppressor proteins, sprouty 1 and sprouty 2 are deregulated in breast cancer. Cancer Res 2004; 64:6127-36.
22. Wang L, Ji C, Wu H, Xu J, Wu Q, Dai J, et al. Identification and expression analysis of a novel splice variant of human Sprouty1 gene. Int J Mol Med 2003; 12:783-7.
23. Jones PA, Taylor SM. Cellular differentiation, cytidine analogs and DNA methylation. Cell 1980; 20:85-93.
24. Lee WH, Morton RA, Epstein JI, Brooks JD, Campbell PA, Bova GS, et al. Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. Proc Natl Acad Sci USA 1994; 91:11733-7.
25. Colella S, Shen L, Baggerly KA, Issa JP, Krahe R. Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. Biotechniques 2003; 35:146-50.
26. Kwabi-Addo B, Chung W, Shen L, Ittmann M, Wheeler T, Jelinek J, Issa JP. Age-related DNA methylation changes in normal human prostate tissues. Clin Cancer Res 2007; 13:3796-802.
27. Wheeler TM, Lebovitz RM. Fresh tissue harvest for research from prostatectomy specimens. Prostate 1994; 25:274-9.

NKX2-5, a Potential Tumor Suppressor Gene is Down-Regulated in Prostate Cancer.

Daremipouran M[1], Wang S[1], Ellis A[1], Bamji Z[1], Abajomi B[1], Ittmann M[2], Kwabi-Addo B[1*].

[1]*Howard University Cancer Center, 2041 Georgia Ave N.W., Washington D.C. 20060 USA.*

[2]*Department of Pathology, Baylor College of Medicine, 1 Baylor Plaza, Houston TX 77030 USA.*

RUNNING TITLE: Expression and Regulation of NKX2-5 in Prostate Cancer Cells

KEY WORDS: NKX2-5, immunohistochemistry, RT-PCR, shRNA, and prostate cancer

*Correspondence:

Bernard Kwabi-Addo, PhD,

Howard University Cancer Center

2041 Georgia Ave. N.W. Washington D. C. 20060

Tel: 202 806 9149

Fax: 202 667 1686

E-mail: bkwabi-addo@howard.edu

Abstract

Prostate cancer (PCa) is a common malignancy and a leading cause of cancer deaths among men in the United States. Abundant evidence has accumulated to suggest that epigenetic DNA methylation changes may appear earlier during PCa development than genetic changes, as well as more commonly and consistently. Recently, we have identified NKX2-5 as a novel gene that is hypermethylated in PCa. To identify the biological role of NKX2-5 in human prostate cancer, we carried out gain-and-loss functional studies of NKX2-5 in prostate cancer cell lines and validated expression at the RNA transcript level using quantitative RT-PCR. Over-expression of NKX2-5 was detrimental to prostate cancer cell proliferation as evidenced by significant inhibition of prostate cancer cell proliferation in comparison to vector only control and this is due to cell arrest in G_o/G_1 phase and increase apoptosis. In contract, successful knockdown of NKX2-5 by shRNA transfection increased prostate cancer cell proliferation. Western blot analysis of NKX2-5 signaling demonstrates that NKX2-5 plays a key regulatory role in the expression of several important genes including p53, PTEN, and the androgen receptor. Our observations indicate that NKX2-5 is a potential tumor suppressor gene that is frequently inactivated in prostate cancer. Because this gene plays important role in several signal transduction pathways, it can be exploited as potential biomarker for the early detection of prostate cancer and could be an attractive target to explore for drug investigation or gene therapies of prostate cancer.

Introduction:

Prostate cancer (PCa) is the second most common malignancy and the second leading cause of cancer deaths in men in the United States [1,2]. Despite improvements in diagnosis and treatment, it remains the second leading cause of cancer deaths in US men [2]. This is in part because the molecular mechanisms of prostate cancer development and progression remains poorly understood. For prostate cancer, no consistent cancer pathway is known as for other malignancies such as colorectal cancer. Instead, recent evidence suggests that prostate cancer cells carry a variety of genetic defects, including gene mutations, deletions, chromosomal rearrangements and epigenetic changes in DNA methylation [3]. Epigenetic inactivation of genes in cancer cells is largely based on transcriptional silencing by aberrant CpG methylation of CpG-rich promoter regions [4,5]. For human PCa, abundant evidence has accumulated to suggest that somatic epigenetic alterations such as DNA methylation may appear earlier during cancer development than other genetic alterations, and they also are more common and occur more consistently [6]. The aberrant promoter methylation of GSTP1, encoding the π-class glutathione *S*-transferase (GSTP1), an enzyme capable of detoxifying electrophilic and oxidant carcinogens remains the most common somatic genome abnormality of any kind (>90% of cases) reported thus far for PCa, appearing earlier and more frequently than other gene defects that arise during PCa development [7] suggesting that CpG hypermethylation may be particularly important in prostate carcinogenesis [8,9]. We have recently reported the hypermethylation of NKX2-5 gene in human prostate cancer [10,11]. NKX2-5 is methylated in prostate and colon cancer, but rarely in breast cancer, suggesting tissue specific differences in the methylation of NKX2-5 [10]. NKX2-5 belongs to a family of homeobox genes that encodes a class of transcription factors

regulating the expression of target genes in a time- and spatial-dependent manner. The *NKX*2-5 gene was initially identified as a mammalian homologue of the Drosophila *tinman* gene [12,13]. The *NKX*2-5 gene is an evolutionarily conserved transcription factor that is required for the organogenesis of the heart [14] and several mutations in *NKX*2-5 have been reported in patients with a variety of cardiac anomalies, including atrioventricular (AV) conduction delays, atrial septal defects, TOF, and DORV [15–17], further underscoring the importance of NKX2-5 in cardiac development. However, very little is known about the role of NKX2-5 in cancer development. One paper described a role for NKX2-5 in ovarian cancer suggesting a potential molecular target for NKX2-5 in the treatment of ovarian cancer [18]. The human *NKX*2-5 gene maps to chromosome 5q34 [19] and different groups have reported several chromosomal rearrangement involving the *NKX*2-5 locus on 5q34 in T-cell acute lymphoblastic leukemia [20–22] support an important role for NKX2-5 in malignant transformation. One target of this chromosomal loss could be NKX2-5 gene.

We have found NKX2-5 to be specifically hypermethylated in the prostate tumors when compared with the normal prostate tissues. NKX2-5 seems to be a novel frequent cancer-associated hypermethylated CpG island in prostate cancer, the hypermethylation of which is associated with neoplastic transformation unlike RARβ2, RASSF1A, and GSTP1 which are hypermethylated in premalignant prostate tissues in an age-dependent manner. Therefore, the inclusion of NKX2-5 as a new marker in a panel of hypermethylated genes in prostate cancer can potentially increase the sensitivity and specificity of prostate cancer detection. Our data suggest that not only is the methylation of *NKX2-5* suitable as a marker for PCa detection, but it may also have increased sensitivity for detecting PCa in AA [23]. However, there is very little information about the biological role of NKX2-5 in prostate cancer. In the present study we sought to

investigate the biological role of gain or loss of NKX2-5 expression in human prostate cancer cell lines.

Materials and Methods:

Cell Culture and Human Prostate Tissue Samples

The human prostate cancer cell lines PC3, DU145 and LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA). The immortalized normal prostate epithelial cell line PNT1a was obtained from the European Collection of Cell Cultures (Salisbury, U.K). All cell lines were maintained in complete medium consisting of RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Invitrogen). All human prostate tissue samples were obtained with informed consent and maintained by the Baylor Specialized Program of Research Excellence (SPORE) in the prostate tissue bank [24]. Fresh frozen tissue punches of normal and tumor tissue were obtained at the time of radical prostatectomy. The pathological statuses were confirmed before processing, and the tumor samples had a tumor cell percentage of 70%-100% with Gleason scores of 6-8.

Tissue Microarrays and Immunohistochemistry

Cores (0.6-mm in size) of cancer and uninvolved prostate tissue were obtained from radical prostatectomy specimens and used in immunohistochemistry analysis as previously described [25]. Briefly, antigen retrieval was performed for 30 min in a rice cooker in 10 mM citrate buffer (pH 6.0). Endogenous biotin and peroxidase were blocked using appropriate kits from Vector Laboratories (Burlingame, CA) according to the manufacturer's protocol. Goat polyclonal anti-

NKX2-5 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) was incubated with each tissue array section at 5 ng/ml at 4°C overnight followed by the avidin-biotin peroxidase complex procedure (Vector Laboratories) and counterstaining with hematoxylin as described previously. Slides were then scanned using a Bliss automated slide scanner system to produce high-resolution digital images. Staining was evaluated in the normal and prostate cancer epithelial cells as described previously. Staining intensity was graded as absent (0), weak (1+), intermediate (2+), or strong (3+). The extent of staining was estimated and scored as follows: no staining (0); 1–33% of cell stained (1+); 34–66% of cell stained (2+); or 67–100% of cells stained (3+). The staining index for each case was then calculated by multiplying the average intensity score for the three cores by the average percentage score for the three cores, yielding a 10-point staining index ranging from 0 (no staining) to 9 (extensive, strong staining) for each case.

Cell transfections

Prostatic normal and cancer cell lines were seeded at 5 X 10^4 cells/well in a 24-well plate in complete medium for 24 hours. Next, cells were transiently transfected with a mammalian expression vector containing the full-length wild-type *NKX*2-5 or empty control vector (pCMV6). For down-regulation of NKX2-5 by RNA interference experiments, shRNA plasmids for human NKX2-5 (pRFP-C-RS, Origene) were used or a plasmid expressing shRNA that does not much any human, mouse or rat gene was included as control. Cells were transfected over night with 0.8 μg DNA and 2 μl Lipofectamine 2000 or LTX (Invitrogen, Carlsbad, CA) in complete medium without antibiotics. After 24, 48, or 72 hr cell were trypsinized and counted

using a Coulter counter (Beckman Coulter Z Series Coulter Counter). A second transfected plate was used to collect RNA and protein extracted for expression analysis at the same time.

Primer Design and Synthesis for Real-Time PCR

Oligonucleotide primers for NKX-2.5 were forward, 5`- CACGTCCACGCAGGTCAAG-3`, and reverse, 5`- GCCCCACCAGCTCCAGAG-3`; for β-actin were forward, 5`-AGCACGGCATCGTCACCAACT-3`, and reverse 5`-TGGCTGGGGTGTTGAAGGTCT-3` and probe 5`-CTGGTCCTGCCGCTGCCGCTTG-3`; for p53 were forward, 5`-CTCCTCAGCATCTTATCCGAGTG-3`, and reverse, 5`- GGTGGTACAGTCAGAGCCAAC -3` and probe 5`- AGGCGGCTCATAGGGCACCACCA -3`; for PTEN were forward, `5-ACCCACCACAGCTAGAACTTATC-3`, and reverse , 5`-ATTTGCCCCGATGTAATAAATATGC -3`and probe 5`-TTACACCAGTTCGTCCCTTTCCAGC -3`; and for Histone H1 were forward, 5`-TGCCACGCCCAAGAAAGC -3`, and reverse, 5`- CTTCTTGCCGGCCCTCTTG-3` an probe 3`- ACCCAAGACTGTCAAAGCCAAGCCG -5`; and for androgen receptor (AR) were forward 5`- CCTCCCGGCGCCAGTTTG -3` and reverse 5`- GAAGGTTGCTGTTCCTCATCCAG -3` and probe, 5`- AGCAGCAGCAGCAGCAGCAGCA-3` and for GSTPI were forward, 5`--3` and revere, and probe 5`--3`. Primers were carefully designed to cross exon/intron regions and to avoid the formation of primer-dimers, hairpins, and self-complementarity. The nucleotide positions for the amplification products as given per the GenBank accession nos. are 707-795, 1312-1464, 256–435, 451-575, 1260-1465, 891-1338, and 594-725 for NKX2-5 (XM_036349), PTEN (NM_000314), β-actin (BC004251), p53 (NM_001126117), AR (NM_000044), and GSTPI (NM_000852) respectively.

cDNA Synthesis and Quantitative Real-Time PCR

Total RNA extracted from cells using TRIzoL Reagent (Invitrogen) was used in first strand DNA (cDNA) synthesis using iScript^{TM} cDNA synthesis kit (Bio-RAD) and according to the manufacturer's protocol. Real-time PCR was carried out in a Bio-RAD iCycler real-time PCR thermal cycler (Bio-Rad, Hercules, CA) as described previously [26] and incorporating the following optimized PCR reaction conditions: The amplification of NKX-2.5 was carried out as follows: a 3 min hot start at 95°C; followed by 40 cycles of denaturation at 95°C for 15 s; and annealing at 61°C for 30 s. The amplification protocol for PTEN was the same as for NKX-2.5 except that annealing was done at 60°C. The amplification protocol for p53, β-actin, AR, Histone H1, and GST-pi was carried out as follows: a 3 min hot start at 95°C; followed by 40 cycles of denaturation at 95°C for 30 s; annealing at 56°C for 20 s; and a 72°C extension for 30 s. Each experiment was done in duplicate. The threshold cycle (C_t) values in log linear range representing the detection threshold values were used for quantitation and expressed as copy numbers based on a standard curve generated using plasmid DNA.

Hormonal induction

For hormonal induction, LNCaP cells were grown for 48 h in RPMI containing 2% FBS that was charcoal-treated (CT, Invitrogen) to remove steroids, followed by an additional 24 h in RPMI containing 0.5% CT-FBS. The synthetic ligands; testosterone (10^{-8}M), 17β-estradiol (10^{-8}M), progesterone (10^{-8}M) and dexamethasone (10^{-7}M) and vehicle (EtOH) were each added to cells maintained as above and treated for 24 h. All compounds were purchased from Sigma Aldrich. Total RNA was then prepared and used in RT-PCR as described above.

Western Blot Analysis

Total protein was extracted from cells using RIPA Buffer (Thermo Scientific) and nuclear and cytoplasmic extracts were extracted with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer's instructions. For Western blots, 40 μg of total protein extract or 15 μg of nuclear protein was used in standard Western blot analysis. The primary antibodies were as follows: anti-NKX-2.5 goat polyclonal antibody (1:100), anti-p53 (1:100) mouse monoclonal antibody, anti-PTEN (1:100) mouse monoclonal antibody, anti-AR (androgen receptor, 1:50), rabbit polyclonal antibody, anti-Histone H1 (1:50) mouse monoclonal antibody and anti-β-actin (1:5000; Sigma). The corresponding horseradish peroxidase-labeled secondary antibodies were as follows: mouse anti-goat IgG (1:5000), bovine anti-mouse IgG (1:5000) or mouse anti-rabbit IgG (1:1000) secondary antibodies. The antigen-antibody reaction was detected with SuperSignal West Femto chemoluminecence (ECL) assay (Thermo Scientific) and a Gel Doc™ XR+ System (Biorad). All antibodies were purchased from Santa Cruz Biotechnologies unless otherwise stated.

Cell Cycle and Apoptosis Analysis

Prostate cancer cells transfected with *NKX*2-5 expression vector or the empty vector were harvested and stained with propidium iodide (Invitrogen; according to the manusfacturer's instruction) and analyzed using a counting chamber (Nexelom Bioscience). To discriminate between viable and nonviable (dead/apoptotic) cells, Annexin-PE assay was performed, and percentage apoptosis estimated with Cellometer Vision (Nexelom Bioscience). To determine the cell cycle distribution, prostate cancer cells were transfected with NKX2-5 expression vector or

the empty vector only. Cells were harvested and stained with propidium iodide as described above and analyzed by Flow Cytometry using BD FACS Calibur flow cytometer according to manufacturer's instruction.

Results

Expression of NKX2-5 in Normal and Neoplastic Human Prostate Tissue

To gain insight into the cellular localization of NKX2-5 in human prostate tissues, we assessed NKX2-5 expression in 25 pairs of matched benign and prostate cancer tissues using immunohistochemistry. For both normal and prostate cancer tissues, we observed variable NKX2-5 staining in the cytoplasm as well as the nucleus (Fig 1a). The cytoplasmic staining was higher than the nuclear staining (Table 1). Overall, we observed the cytoplasmic staining to be statistically less in cancers than the normal control ($p=0.034$) but not for nuclear staining ($p=0.886$). Because NKX2-5 appears to be localized in both the nucleus and cytoplasm, we wanted to know whether it is regulated by hormones. We therefore treated LNCaP cells with various ligands for 24 hr, isolated total RNA and analyzed NKX2-5 expression by RT-PCR (Fig 1b). Results showed approximately 5 fold, 8 fold, 3 fold, and 3 fold increase in NKX2-5 expression in response to testosterone, 17β-estradiol, progesterone and dexamethasome respectively, suggesting that NKX2-5 expression can be regulated by multiple hormonal signals in prostate cancer cells.

To determine whether NKX2-5 is decreased in human prostate cancer, we measured the expression of NKX2-5 in normal and neoplastic prostatic epithelium using quantitative RT-PCR analysis with mRNA from a total of 40 pairs of matched normal and tumor prostate tissue samples. We used β-actin as an endogenous mRNA control. The real-time data is presented as

the ratio of NKX2-5 X 10^3/β-actin transcript for the samples analyzed (Fig. 1c). The expression of NKX2-5 in both normal and cancer tissues were variable, presumably reflecting both random variability in tissue composition (i.e., epithelial content) and variable expression per cell. However, NKX2-5 expression was approximately 2 fold higher in the normal prostate tissues (5.778 $\pm$ 1.005, SEM) compared to prostate cancer (3.402 $\pm$ 0.6293, SEM, $p < 0.001$). Examination of paired normal versus cancer tissues revealed decreased NKX2-5 expression in 23 out of 38 cancer cases (60%) relative to matched normal tissues.

Effect of gain-or-loss of NKX2-5 Expression in Human Prostate Cancer Cells

To determine the biological effect of NKX2-5 expression in human prostate cancer cells, we adopted a gain-and-loss of functional approach. When prostate cancer cells were transiently transfected with pCMV6- NKX2-5 (encoding the full length of NKX2-5 sequence) we observed approximately 37%, 36% and 38% inhibition of LNCaP, DU145 and PC-3 cell proliferation respectively, over a 3 day period when compared to cells transfected with the vector only (Fig. 2a). Analysis of NKX2-5 expression in the same cells by Western blotting showed increase in NKX2-5 expression in cells transfected with the NKX2-5 expressing plasmid compared to the vector only transfection (Fig 2b). In contrast, when we endogenously knock-down NKX2-5 expression by transfecting prostate cancer cells with shRNA plasmids for human NKX2-5, we observed 89%, 50% and 60% increase in LNCaP, DU145 and PC-3 cell proliferation after 24 hours of transfection in comparison with cells transfected with scrambled oligonucleotide only (Fig. 2c). To demonstrate a consecutive down-regulation of NKX2-5, Western blot analysis was performed (Fig 2d). The results demonstrates that forced expression of NKX2-5 can significantly inhibit prostate cancer cell proliferation whereas a modest decrease in NKX2-5 protein can

significantly increase prostate cancer cell proliferation clearly suggesting that NKX2-5 plays a role in prostate cancer cell proliferation.

NKX2-5 induces G_0/G_1 cell cycle arrest and promotes apoptosis.

We further examined how NKX2-5 might affect cell cycle progression and cell proliferation. Using transient transfection of prostate cancer cell lines with either the NKX2-5 expression plasmid or an empty vector plasmid, we compared their cell cycle distributions. The percentage of G_0/G_1 phase cells in NKX2-5 transfected cells was approximately 30% more than that in the empty vector only transfected cells, whereas NKX2-5 transfections showed less S or G_2/M phase cells than the control transfections (Fig 3a; because we observed similar responses in all 3 prostate cancer cell lines, we are only showing the response in DU145 cells). Next we determined whether NKX2-5 could affect apoptosis in prostate cancer cell lines. Using Annexin V/PI apoptotic assay, we observed about 3-fold increase apoptosis in prostate cancer cells transfected with the NKX2-5 plasmid when comparison with the vector only transfection (Fig 3b). Collectively, these findings indicate that NKX2-5 can cause cell cycle arrest and is a pro-apoptotic factor for prostate cancer cell proliferation.

NKX2-5 Regulates the Expression of p53, PTEN, Histone H1, and AR in Human Prostate Cancer Cell Lines.

To investigate the involvement of NKX2-5 expression in prostate carcinogenesis, we investigated the expression levels of several target genes that are important in prostate cancer, in response to either gain-or-loss of NKX2-5 expression. Western blot analysis demonstrates the increase expression of AR, p53, PTEN, and SOD1 proteins in all prostate cell lines transfected

with NKX2-5 plasmid in comparison with empty vector (Fig 4a). We did not see any significant effect of increased NKX2-5 expression on GSTP1 protein level. However, we observed increased expression of GSTPI mRNA transcript in PNT1a and DU145 cells and somewhat reduced expression in PC3 cells by RT-PCR analysis (data not shown). In contrast, we observed reduced expression of AR, p53, PTEN, SOD1 and a modest reduction of GSTPI expression in the prostate cells transfected with NKX2-5 shRNA plasmid in comparison with scrambled oligonucleotide transfections (Fig 4b). Taken together, our data indicate an important regulatory role for NKX2-5 expression in prostate cancer cells by targeting several regulatory proteins.

Discussion

Very little is known about the involvement of NKX2-5 in human prostate cancer. We have recently identified NKX2-5 to be hypermethylated in prostate cancer tissues [10,11,23]. The inactivation of gene expression by DNA methylation constitutes an important mechanism by which cancer can grow in an uncontrolled way [27]. In the present study, we have found that NKX2-5 an important transcription factor that is required for the organogenesis of the heart [14] and is down-regulated in about 60% of human prostate cancer tissues when compared with normal prostate tissue in the same patient samples. It is not surprising that only a fraction of prostate cancers show decreased NKX2-5. All epithelial malignancies have a variety of genetic and epigenetic alterations and in general, only a fraction of cases of a given tumor type have a specific alteration. For example, only a subset of prostate cancer tissues has alterations of the NKX3.1 gene, another member of the NK class of homeodomain proteins [28]. Much work has been done on NKX3.1 whose expression in human was primarily detected in the testis, in rare mucous glands of the lung and ureteral epithelial cells, but the strongest expression was found in

prostate epithelia [29]. A potential tumor suppressor role has been proposed for NKX3.1 in human prostate cancer, due to frequent allelic loss of the gene that accompanies initiation of prostate cancer [30,31]. However, no mutations occur in the coding region which might inactivate its presumed tumor suppressor function [32] and some particularly aggressive cancers over-express the protein [33]. Recent findings suggest a function of NKX3.1 in protection against oxidative damage involved in prostate carcinogenesis [29,34], thus NKX3.1 may act differently at various stages of prostate cancer. Nevertheless, NKX3.1 is often referred to as candidate tumor suppressor gene.

Our current observation suggests a potential tumor suppressor role for NKX2-5 in human prostate cancer cells. First of all, NKX2-5 appears to be down-regulated in human prostate cancer tissues in comparison with normal prostate tissues. Secondly, over-expression of NKX2-5 leads to growth arrest and increased apoptosis. Furthermore, NKX2-5 appears to modulate the expression of p53, PTEN, AR, and GSTP1, genes found to be altered in a significant proportion of prostate cancer [35]. In addition, NKX2-5 also modulate the expression of superoxide dismutase 1 (SOD1) in prostate cancer cells, to indicate that NKX2-5 may play a role in prostate cells response to oxidative stress [29,34,36]. We have found that NKX2-5 protein is localized in the cytoplasm and nucleus in both normal and prostate cancer tissues, although in the cancer tissues. It is not clear why a putative transcription factor with nuclear activity would be localized in the cytoplasm. We reason that the protein may potentially be held in an inactive state in the cytoplasm and get shuffled into the nucleus in response to some yet unidentified stimulus. Activation of NKX2-5 expression by various hormones suggests that this could potentially be involved in the shuffling of NKX2-5 from the cytoplasm into the nucleus. Ongoing studies in the

laboratory are to elucidate the molecular mechanisms regulating NKX2-5 subcellular localization and identify the factors that control its shuffling from the cytoplasm into the nucleus.

In conclusion, the finding that NKX2-5 control cell growth and differentiation and that it also regulate the expression of several signaling intermediates important in prostate carcinogenesis suggests that the alterations of NKX2-5 expression affect human prostate carcinogenesis. Thus, manipulating NKX2-5 expression may serve as a therapeutic strategy for prostate cancer.

Acknowledgements:

This work was supported by grant from the Department of Defense Prostate Cancer Research Program Idea Award; PC073828 to Bernard Kwabi-Addo. This work is also supported by the use of facilities of Howard University Cancer Center and Baylor College of Medicine.

Reference List

1. Gregorakis AK, Holmes EH, Murphy GP (1998) Prostate-specific membrane antigen: current and future utility. Semin Urol Oncol 16: 2-12.
2. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ (2005) Cancer statistics, 2005. CA Cancer J Clin 55: 10-30. 55/1/10 [pii].
3. Nelson WG, De Marzo AM, Isaacs WB (2003) Prostate cancer. N Engl J Med 349: 366-381. 10.1056/NEJMra021562 [doi];349/4/366 [pii].
4. Feinberg AP, Tycko B (2004) The history of cancer epigenetics. Nat Rev Cancer 4: 143-153. 10.1038/nrc1279 [doi];nrc1279 [pii].
5. Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. Nat Rev Genet 3: 415-428. 10.1038/nrg816 [doi];nrg816 [pii].

6. Nelson WG, Yegnasubramanian S, Agoston AT, Bastian PJ, Lee BH, Nakayama M, De Marzo AM (2007) Abnormal DNA methylation, epigenetics, and prostate cancer. Front Biosci 12: 4254-4266. 2385 [pii].

7. Nelson WG, De Marzo AM, Isaacs WB (2003) Prostate cancer. N Engl J Med 349: 366-381.

8. Zingg JM, Jones PA (1997) Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. Carcinogenesis 18: 869-882.

9. Gonzalgo ML, Liang G, Spruck CH, III, Zingg JM, Rideout WM, III, Jones PA (1997) Identification and characterization of differentially methylated regions of genomic DNA by methylation-sensitive arbitrarily primed PCR. Cancer Res 57: 594-599.

10. Chung W, Kwabi-Addo B, Ittmann M, Jelinek J, Shen L, Yu Y, Issa JP (2008) Identification of novel tumor markers in prostate, colon and breast cancer by unbiased methylation profiling. PLoS One 3: e2079. 10.1371/journal.pone.0002079 [doi].

11. Kwabi-Addo B, Chung W, Shen L, Ittmann M, Wheeler T, Jelinek J, Issa JP (2007) Age-related DNA methylation changes in normal human prostate tissues. Clin Cancer Res 13: 3796-3802. 13/13/3796 [pii];10.1158/1078-0432.CCR-07-0085 [doi].

12. Bodmer R (1993) The gene tinman is required for specification of the heart and visceral muscles in Drosophila. Development 118: 719-729.

13. Komuro I, Izumo S (1993) Csx: a murine homeobox-containing gene specifically expressed in the developing heart. Proc Natl Acad Sci U S A 90: 8145-8149.

14. Harvey RP, Lai D, Elliott D, Biben C, Solloway M, Prall O, Stennard F, Schindeler A, Groves N, Lavulo L, Hyun C, Yeoh T, Costa M, Furtado M, Kirk E (2002) Homeodomain factor Nkx2-5 in heart development and disease. Cold Spring Harb Symp Quant Biol 67: 107-114.

15. Benson DW, Silberbach GM, Kavanaugh-McHugh A, Cottrill C, Zhang Y, Riggs S, Smalls O, Johnson MC, Watson MS, Seidman JG, Seidman CE, Plowden J, Kugler JD (1999) Mutations in the cardiac transcription factor NKX2.5 affect diverse cardiac developmental pathways. J Clin Invest 104: 1567-1573. 10.1172/JCI8154 [doi].

16. Hosoda T, Komuro I, Shiojima I, Hiroi Y, Harada M, Murakawa Y, Hirata Y, Yazaki Y (1999) Familial atrial septal defect and atrioventricular conduction disturbance associated with a point mutation in the cardiac homeobox gene CSX/NKX2-5 in a Japanese patient. Jpn Circ J 63: 425-426.

17. Schott JJ, Benson DW, Basson CT, Pease W, Silberbach GM, Moak JP, Maron BJ, Seidman CE, Seidman JG (1998) Congenital heart disease caused by mutations in the transcription factor NKX2-5. Science 281: 108-111.

18. Shibata K, Kajiyama H, Yamamoto E, Terauchi M, Ino K, Nawa A, Kikkawa F (2008) Establishment and characterization of an ovarian yolk sac tumor cell line reveals possible involvement of Nkx2.5 in tumor development. Oncology 74: 104-111. 000139138 [pii];10.1159/000139138 [doi].

19. Shiojima I, Komuro I, Inazawa J, Nakahori Y, Matsushita I, Abe T, Nagai R, Yazaki Y (1995) Assignment of cardiac homeobox gene CSX to human chromosome 5q34. Genomics 27: 204-206. S0888-7543(85)71027-0 [pii];10.1006/geno.1995.1027 [doi].

20. Nagel S, Kaufmann M, Drexler HG, Macleod RA (2003) The cardiac homeobox gene NKX2-5 is deregulated by juxtaposition with BCL11B in pediatric T-ALL cell lines via a novel t(5;14)(q35.1;q32.2). Cancer Res 63: 5329-5334.

21. Nagel S, Scherr M, Kel A, Hornischer K, Crawford GE, Kaufmann M, Meyer C, Drexler HG, Macleod RA (2007) Activation of TLX3 and NKX2-5 in t(5;14)(q35;q32) T-cell acute lymphoblastic leukemia by remote 3'-BCL11B enhancers and coregulation by PU.1 and HMGA1. Cancer Res 67: 1461-1471. 67/4/1461 [pii];10.1158/0008-5472.CAN-06-2615 [doi].

22. Przybylski GK, Dik WA, Grabarczyk P, Wanzeck J, Chudobska P, Jankowski K, von BA, van Dongen JJ, Schmidt CA, Langerak AW (2006) The effect of a novel recombination between the homeobox gene NKX2-5 and the TRD locus in T-cell acute lymphoblastic leukemia on activation of the NKX2-5 gene. Haematologica 91: 317-321.

23. Kwabi-Addo B, Wang S, Chung W, Jelinek J, Patierno SR, Wang BD, Andrawis R, Lee NH, Apprey V, Issa JP, Ittmann M (2010) Identification of differentially methylated genes in normal prostate tissues from African American and Caucasian men. Clin Cancer Res 16: 3539-3547. 1078-0432.CCR-09-3342 [pii];10.1158/1078-0432.CCR-09-3342 [doi].

24. Wheeler TM, Lebovitz RM (1994) Fresh tissue harvest for research from prostatectomy specimens. Prostate 25: 274-279.

25. Kwabi-Addo B, Wang J, Erdem H, Vaid A, Castro P, Ayala G, Ittmann M (2004) The expression of Sprouty1, an inhibitor of fibroblast growth factor signal transduction, is decreased in human prostate cancer. Cancer Res 64: 4728-4735. 10.1158/0008-5472.CAN-03-3759 [doi];64/14/4728 [pii].

26. Polnaszek N, Kwabi-Addo B, Wang J, Ittmann M (2004) FGF17 is an autocrine prostatic epithelial growth factor and is upregulated in benign prostatic hyperplasia. Prostate 60: 18-24. 10.1002/pros.20026 [doi].

27. Esteller M, Sparks A, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Gonzalez S, Tarafa G, Sidransky D, Meltzer SJ, Baylin SB, Herman JG (2000) Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res 60: 4366-4371.

28. Gelmann EP, Steadman DJ, Ma J, Ahronovitz N, Voeller HJ, Swope S, Abbaszadegan M, Brown KM, Strand K, Hayes RB, Stampfer MJ (2002) Occurrence of NKX3.1 C154T polymorphism in men with and without prostate cancer and studies of its effect on protein function. Cancer Res 62: 2654-2659.

29. Bowen C, Bubendorf L, Voeller HJ, Slack R, Willi N, Sauter G, Gasser TC, Koivisto P, Lack EE, Kononen J, Kallioniemi OP, Gelmann EP (2000) Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. Cancer Res 60: 6111-6115.

30. Bethel CR, Faith D, Li X, Guan B, Hicks JL, Lan F, Jenkins RB, Bieberich CJ, De Marzo AM (2006) Decreased NKX3.1 protein expression in focal prostatic atrophy, prostatic intraepithelial neoplasia, and adenocarcinoma: association with gleason score and chromosome 8p deletion. Cancer Res 66: 10683-10690. 66/22/10683 [pii];10.1158/0008-5472.CAN-06-0963 [doi].

31. Ju JH, Maeng JS, Zemedkun M, Ahronovitz N, Mack JW, Ferretti JA, Gelmann EP, Gruschus JM (2006) Physical and functional interactions between the prostate suppressor homeoprotein NKX3.1 and serum response factor. J Mol Biol 360: 989-999. S0022-2836(06)00681-4 [pii];10.1016/j.jmb.2006.05.064 [doi].

32. Voeller HJ, Augustus M, Madike V, Bova GS, Carter KC, Gelmann EP (1997) Coding region of NKX3.1, a prostate-specific homeobox gene on 8p21, is not mutated in human prostate cancers. Cancer Res 57: 4455-4459.

33. Xu LL, Srikantan V, Sesterhenn IA, Augustus M, Dean R, Moul JW, Carter KC, Srivastava S (2000) Expression profile of an androgen regulated prostate specific homeobox gene NKX3.1 in primary prostate cancer. J Urol 163: 972-979. S0022-5347(05)67867-6 [pii].

34. Bowen C, Gelmann EP (2010) NKX3.1 activates cellular response to DNA damage. Cancer Res 70: 3089-3097. 70/8/3089 [pii];10.1158/0008-5472.CAN-09-3138 [doi].

35. Porkka KP, Visakorpi T (2004) Molecular mechanisms of prostate cancer. Eur Urol 45: 683-691. 10.1016/j.eururo.2004.01.012 [doi];S030228380400017X [pii].

36. Bhusari SS, Dobosy JR, Fu V, Almassi N, Oberley T, Jarrard DF (2010) Superoxide dismutase 1 knockdown induces oxidative stress and DNA methylation loss in the prostate. Epigenetics 5: 402-409. 11853 [pii].

Figure Legends

Figure 1- Characterization of NKX2-5 expression in normal and prostate cancer tissues.

A- Localization of NKX2-5 expression in human prostate tissues using immunohistochemical tissue microarrays. Expression of NKX2-5 in normal prostate (I & III) and prostate cancer (II & IV) was determined using immunohistochemical tissue microarrays as described in "Materials and Methods". Staining of cytoplasm and nucleus is detected in all tissues (original magnification, 100x). **B-** NKX2-5 expression is regulated by multiple hormones in LNCaP cells. LNCaP cells were either left untreated or treated with the indicated ligand for 24h. Cells were

harvested, total RNA was prepared and NKX2-5 expression was determined by quantitative RT-PCR analysis using iCycler and expressed relative to GAPDH to correct for variation in the amount of reverse-transcribed RNA. The data is a representative of duplicate experiments. C= control (EtOH); DHT= 5a-dihydrotestosterone; T= testosterone; Prog= progesterone; E2= 17β-estradiol; Dex= dexamethasone. **C**- NKX2-5 expression in normal prostate and prostate cancer as determined by quantitative RT-PCR. NKX2-5 expression in normal prostatic peripheral zone (PZ) and cancer tissues was assessed by quantitiative RT-PCR using a real-time thermal cycler (iCycler; Bio-Rad). NKX2-5 expression levels are displayed as a ratio of NKX2-5 X 10^3 to β-actin transcripts. The NKX2-5 and β-actin values were calculated from standard curves. The data are a representative of duplicate experiments. The mean expression level is indicated. The NKX2-5 expression value from cancer tissues is significantly different from the PZ tissues; $P< 0.05$ (t test) for β-actin normalization.

Figure 2- Function of NKX2-5 in prostate cancer cell lines. A- LNCaP, DU145, and PC3 prostate cancer cells were each transfected with NKX2-5 cDNA cloned into the mammalian expression vector pCMV6 (test) or the vector only (control). At the indicated times after transfection, cells were trypsinized and counted using a Coulter counter. All determinations were performed in triplicate, and the SD is shown. **B**- Protein extracts were collected from LNCaP, DU145, and PC3 cells 2 days post-transfection with vector control transfection (C) or NKX2-5 transfections (T) and analyzed by Western blot with either anti-NKX2-5 antibody or control anti-β-actin antibody. **C**- LNCaP, DU145, and PC3 prostate cancer cells were each transfected with shRNA plasmid to NKX2-5 (test) or scrambled shRNA (control). After 48 hr post-transfection, cells were trypsinized and counted using a Coulter counter and percentage cell proliferation in test experiments expressed relative to control (set at 100%). D- Protein extracts were collected

from LNCaP, DU145, and PC3 cells 2 days post-transfection with scrambled shRNA control (C) or the NKX2-5 shRNA transfections (T) and analyzed by Western blot with either anti-NKX2-5 antibody or control anti-β-actin antibody.

Figure 3: Nkx2-5 induces G0/G1 cell cycle arrest and apoptosis. A. Histogram of FACS cell cycle analysis of DU145 cells transfected with either scrambled shRNA (Ctrl) or shRNA to NKX2-5 (Test). Cells were harvested at 24 and 48 hr post-transfection and stained with propidium iodide. Percentage of cells in G_0/G_1, S and G_2/M phases are shown. **B.** Prostate cell lines PNT1A, DU145, PC3, and LNCaP transfected with either vector only plasmid or NKX2-5 plasmid were harvested at the indicated time points and stained with propidium iodide and apoptosis measured by Annexin-PE assay.

Figure 4- The effect of NKX2-5 on downstream signals. A. The prostate cell lines PNT1A, LNCaP, PC3, and DU145 were with either vector only plasmid (C) or NKX2-5 plasmid (T). **B.** The prostate cell lines were either transfected with scrambled shRNA plasmid (C) or shRNA to NKX2-5 (T). Cells were harvested 24 hr post-transfection and analyzed by Western blot with anti-NKX2-5, anti-GSTP1, anti-SOD1, anti-AR, anti-p53, anti-PTEN antibodies or control anti-β-actin antibody.

Table - Quantitative immunohistochemical analysis of NKX2-5 expression in normal and prostate cancer tissues. Staining intensity ranged from 0 (no staining) to 9 (extensive, strong staining) for each sample. BN-benign prostate tissues; CA- prostate cancer tissues; Cyto-cytoplasmic staining.

Figure 1a

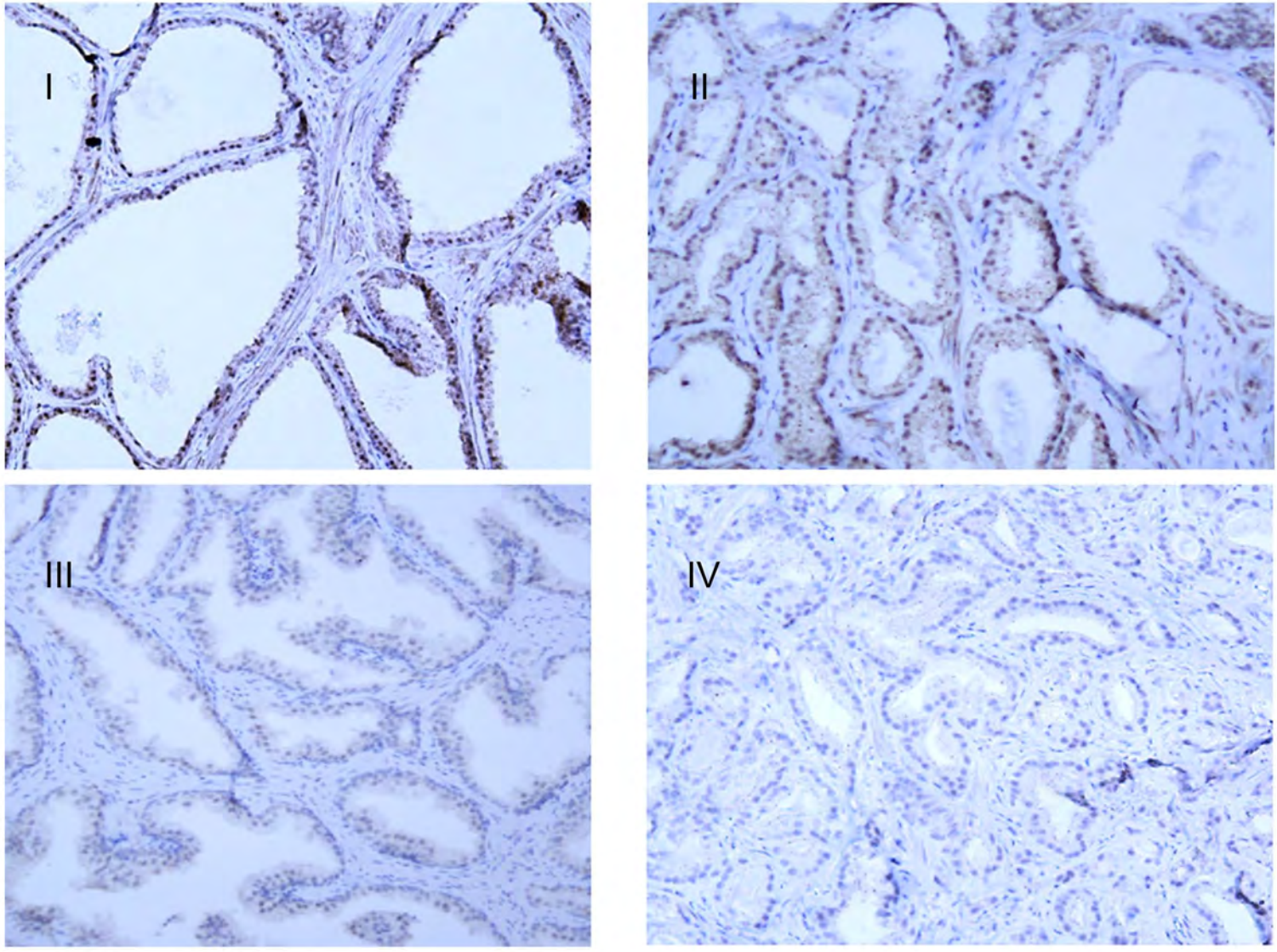


Figure 1b

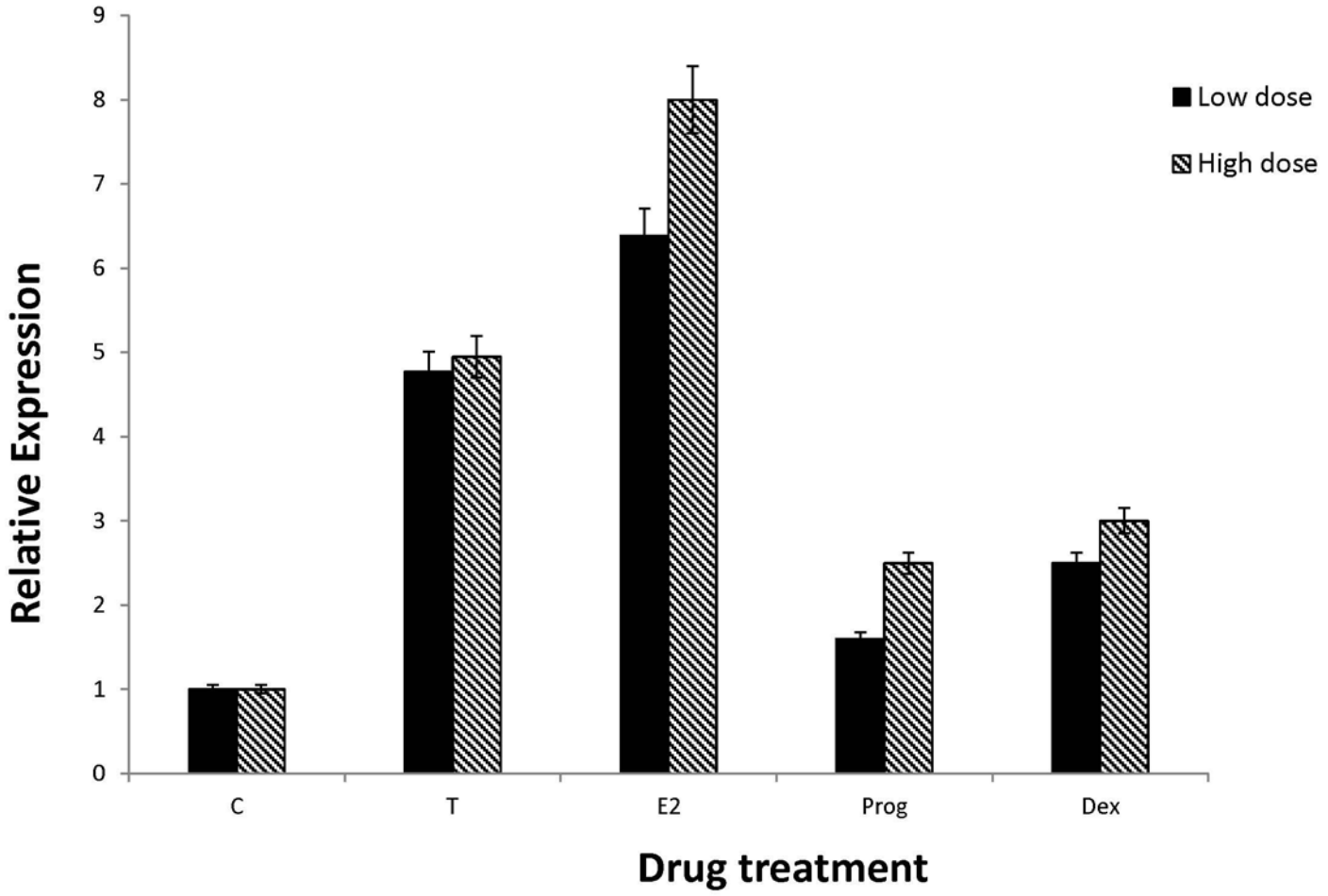

Relative Expression
9
8
7
6
5
4
3
2
1
0
Low dose
High dose
C
T
E2
Prog
Dex
Drug treatment

Figure 1c

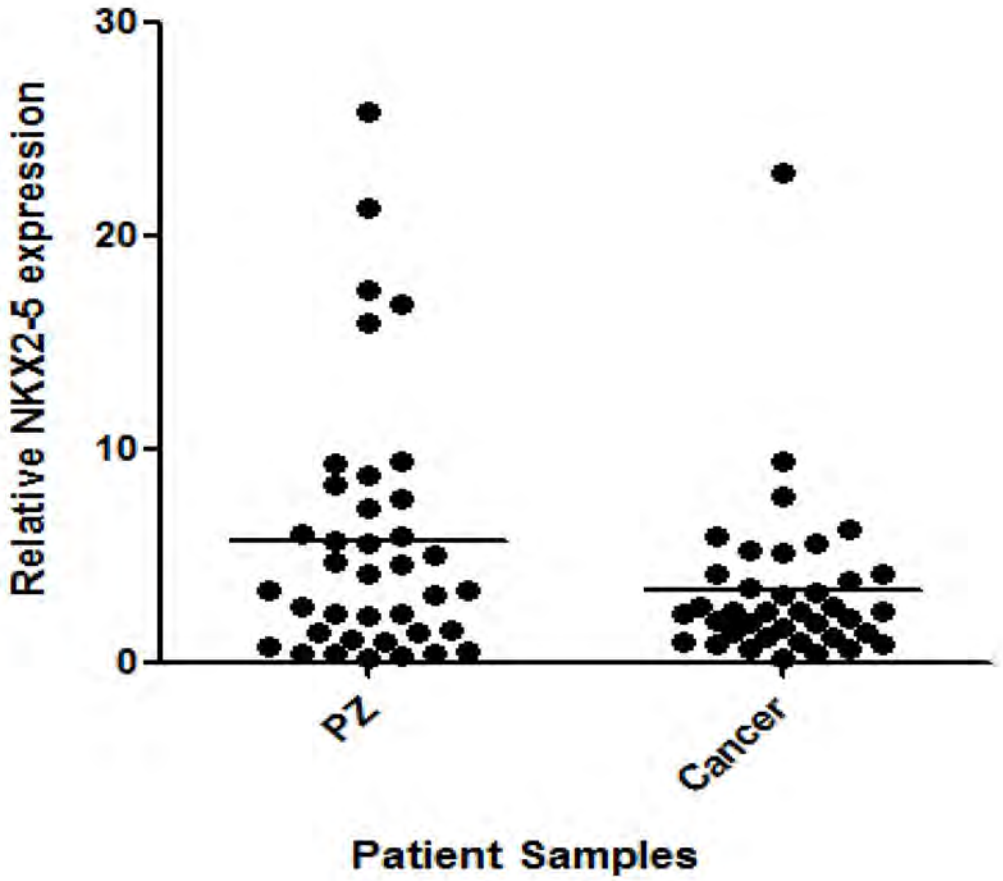

30
20
10
0
Relative NKX2-5 expression
PZ
Cancer
Patient Samples

Figure 2a

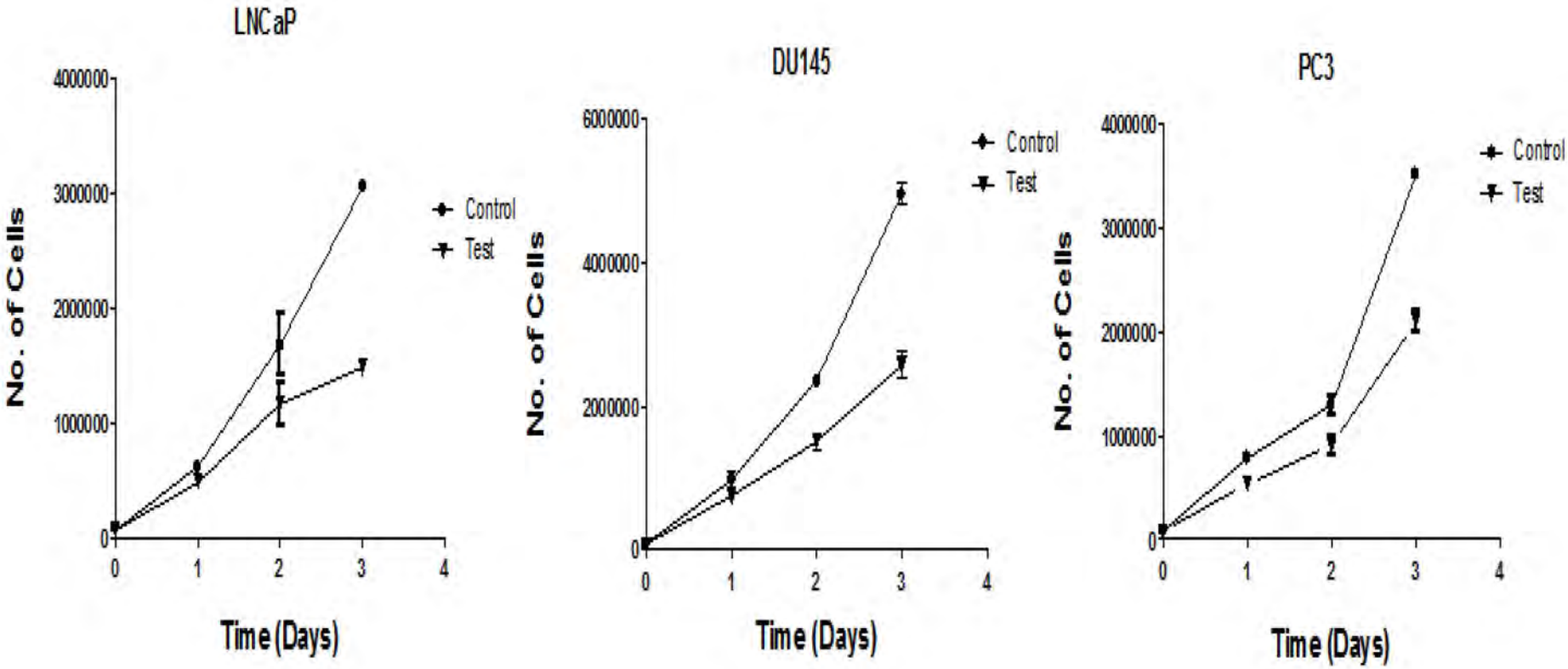

LNCaP
No. of Cells
4000000
3000000
2000000
1000000
0
0
1
2
3
4
Time (Days)
Control
Test
DU145
No. of Cells
6000000
4000000
2000000
0
0
1
2
3
4
Time (Days)
Control
Test
PC3
No. of Cells
4000000
3000000
2000000
1000000
0
0
1
2
3
4
Time (Days)
Control
Test

Figure 2b

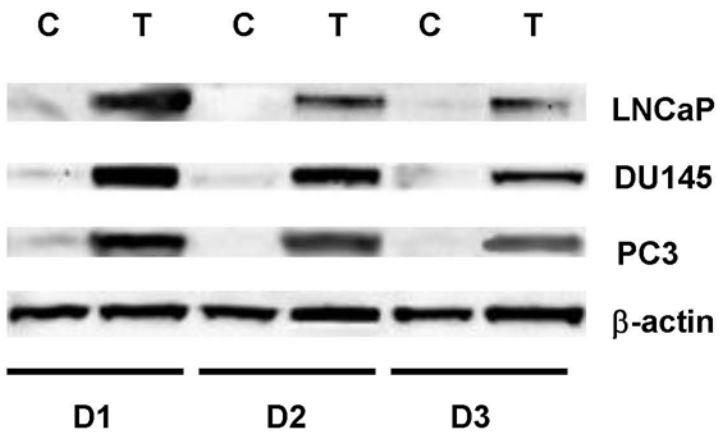


Figure 2c

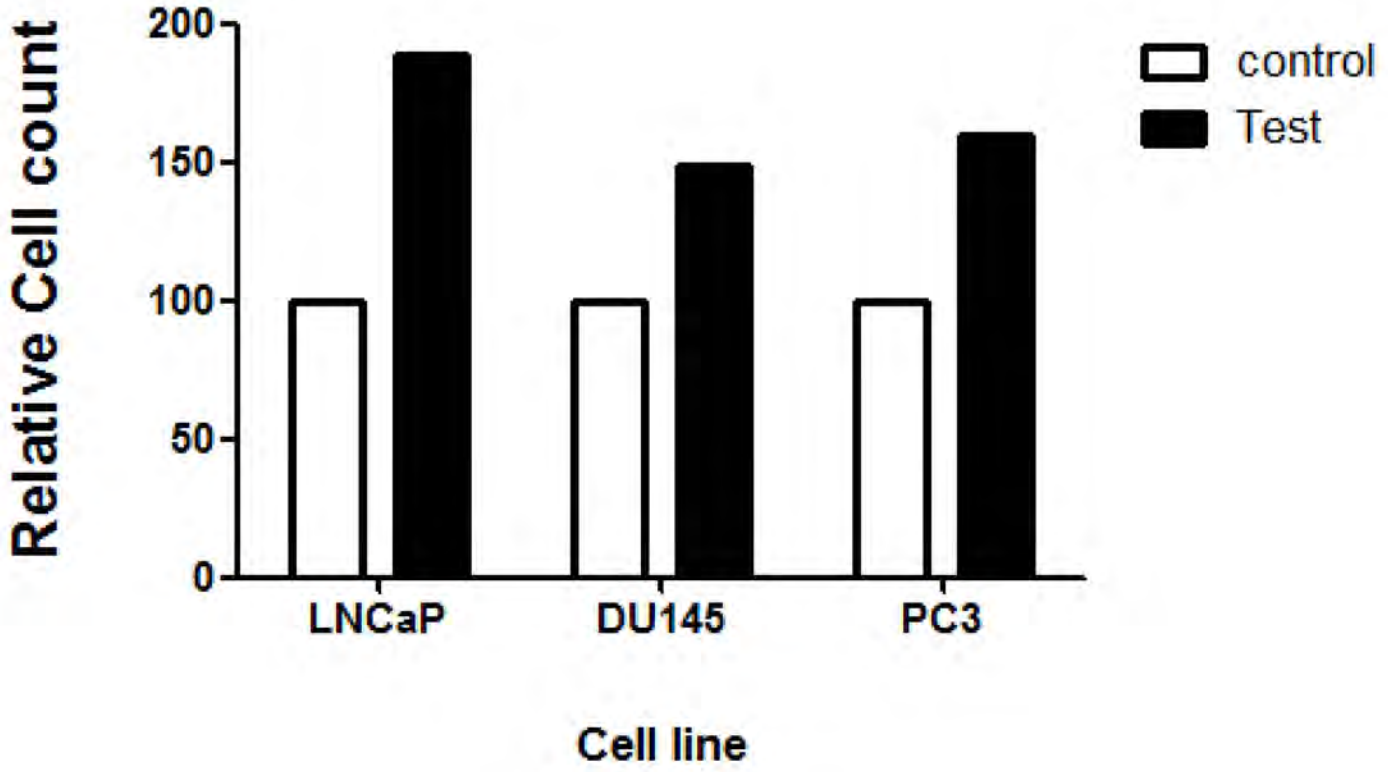

Relative Cell count
200
150
100
50
0
LNCaP
DU145
PC3
Cell line
control
Test

Figure 2d

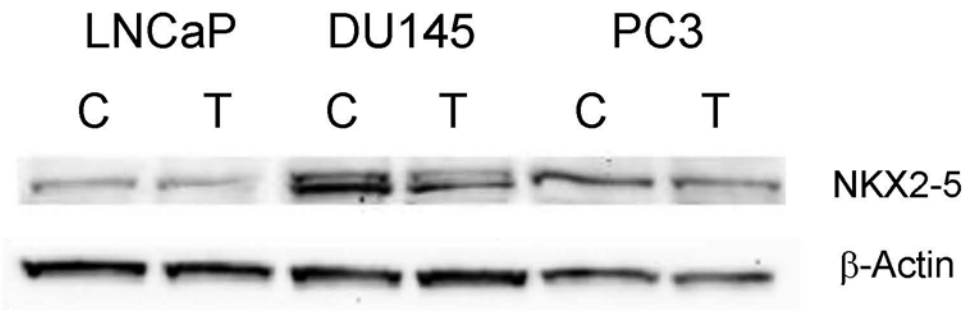


Figure 3a

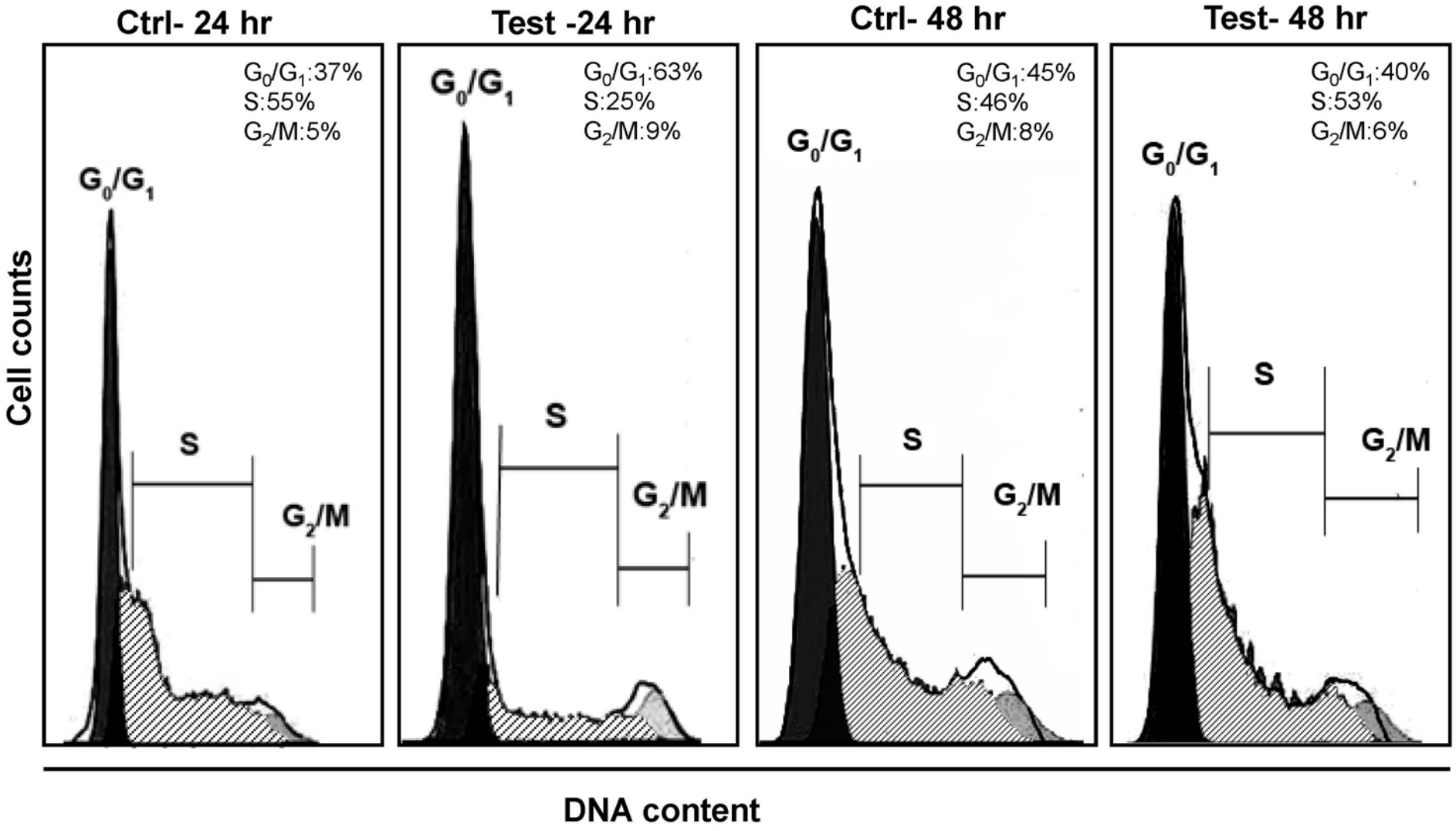
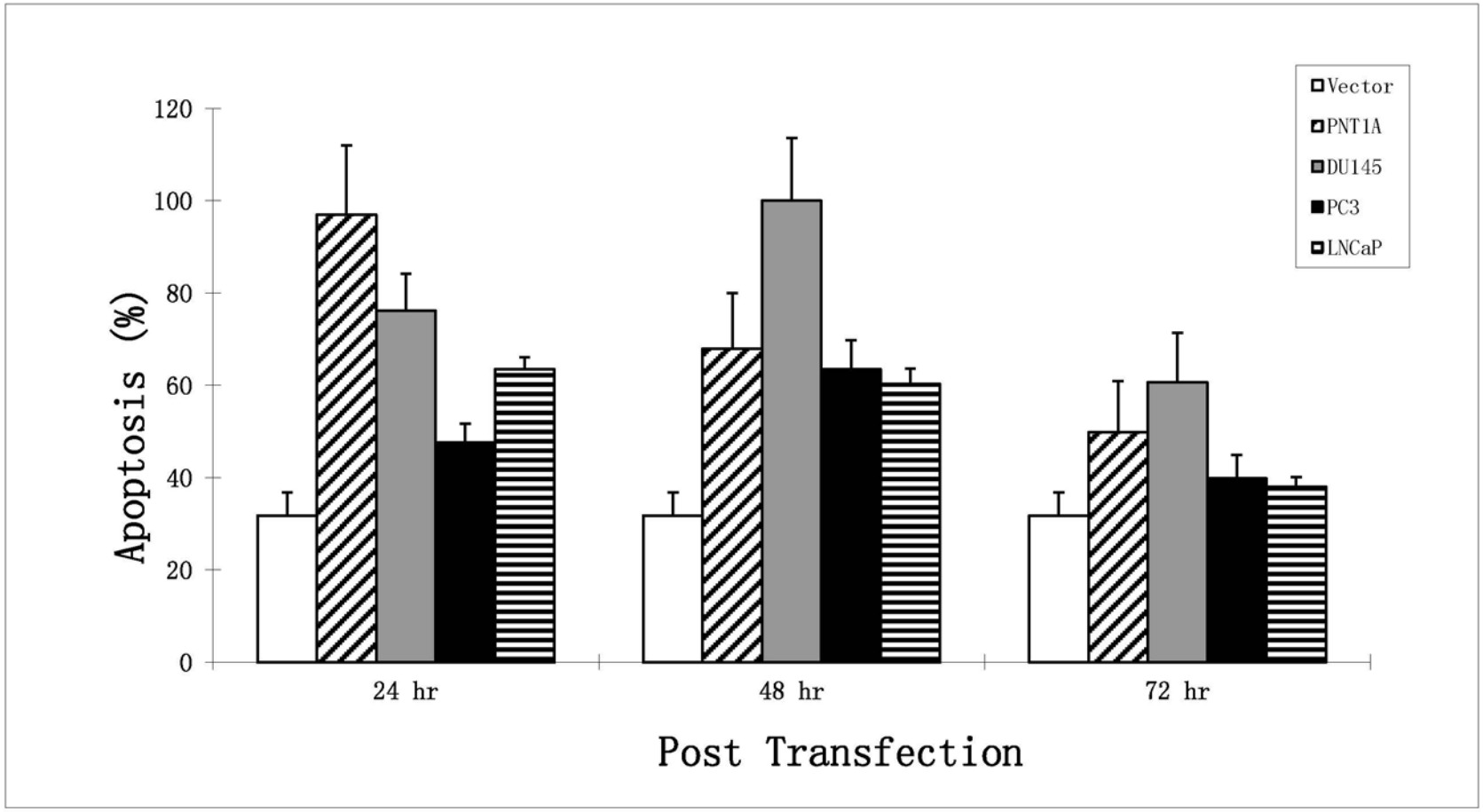

Ctrl- 24 hr
Test -24 hr
Ctrl- 48 hr
Test- 48 hr
G0/G1:37%
S:55%
G2/M:5%
G0/G1:63%
S:25%
G2/M:9%
G0/G1:45%
S:46%
G2/M:8%
G0/G1:40%
S:53%
G2/M:6%
G0/G1
S
G2/M
Cell counts
DNA content

Figure 3b


Vector
PNT1A
DU145
PC3
LNCaP
Apoptosis (%)
120
100
80
60
40
20
0
24 hr
48 hr
72 hr
Post Transfection